

Practitioner's Docket No. 55043

CHAPTER II

**TRANSMITTAL LETTER
TO THE UNITED STATES ELECTED OFFICE (EO/US)
(ENTRY INTO U.S. NATIONAL PHASE UNDER CHAPTER II)**

<u>PCT/IE99/00012</u>	<u>25 February 1999</u>	<u>25 February 1998</u>
INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED

HLA LINKED PRE-ECLAMPSIA AND MISCARRIAGE SUSCEPTIBILITY GENE
TITLE OF INVENTION

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APPLICANTS

Box PCT
Assistant Commissioner for Patents
Washington D.C. 20231
ATTENTION: EO/US

NOTE: To avoid abandonment of the application, the applicant shall furnish to the USPTO, not later than 20 months from the priority date: (1) a copy of the international application, unless it has been previously communicated by the International Bureau or unless it was originally filed in the USPTO; and (2) the basic national fee (see 37 C.F.R. § 1.492(a)). The 30-month time limit may not be extended. 37 C.F.R. § 1.495.

WARNING: Where the items are those which can be submitted to complete the entry of the international application into the national phase are subsequent to 30 months from the priority date the application is still considered to be in the international state and if mailing procedures are utilized to obtain a date the express mail procedure of 37 C.F.R. § 1.10 must be used (since international application papers are not covered by an ordinary certificate of mailing - See 37 C.F.R. § 1.8.

NOTE: Documents and fees must be clearly identified as a submission to enter the national state under 35 USC 371 otherwise the submission will be considered as being made under 35 USC 111. 37 C.F.R. § 1.494(f).

CERTIFICATION UNDER 37 C.F.R. § 1.10*
(Express Mail label number is **mandatory**.)
(Express Mail certification is optional.)

I hereby certify that this paper, along with any document referred to, is being deposited with the United States Postal Service on this date August 25, 2000, in an envelope as "Express Mail Post Office to Addressee," mailing Label Number **EK493902385US**, addressed to the: Assistant Commissioner for Patents, Washington, D.C. 20231.

Deanna M. Rivemider
(type or print name of person mailing paper)

Deanna M. Rivemider
Signature of person mailing paper

WARNING: Certificate of mailing (first class) or facsimile transmission procedures of 37 C.F.R. § 1.8 cannot be used to obtain a date of mailing or transmission for this correspondence

***WARNING:** Each paper or fee filed by "Express Mail" **must** have the number of the "Express Mail" mailing label placed thereon prior to mailing. 37 C.F.R. § 1.10(b).
"Since the filing of correspondence under § 1.10 without the Express Mail mailing label thereon is an oversight that can be avoided by the exercise of reasonable care, requests for waiver of this requirement will **not** be granted on petition." Notice of Oct 24, 1996, 60 Fed Reg 56,439, at 56,442.

1. Applicant herewith submits to the United States Elected Office (EO/US) the following items under 35 U.S.C. 371:
- a. ☒ This express request to immediately begin national examination procedures (35 U.S.C. 371(f)).
 - b. ☒ The U.S. National Fee (35 U.S.C. 371(c)(1)) and other fees (37 C.F.R. § 1.492) as indicated below:

2. Fees

CLAIMS FEE	(1) FOR	(2) NUMBER FILED	(3) NUMBER EXTRA	(4) RATE	(5) CALCULATIONS
[]*	TOTAL CLAIMS	30 - 20 =	10	x \$ 18.00 =	\$ 180.00
	INDEPENDENT CLAIMS	18 - 3 =	15	x \$ 78.00 =	\$1,170.00
	MULTIPLE DEPENDENT CLAIM(S) (if applicable) + \$260.00				\$ 260.00
BASIC FEE**	<input type="checkbox"/> U.S. PTO WAS INTERNATIONAL PRELIMINARY EXAMINATION AUTHORITY Where an International preliminary examination fee as set forth in § 1.482 has been paid on the international application to the U.S. PTO: <input type="checkbox"/> and the international preliminary examination report states that the criteria of novelty, inventive step (non-obviousness) and industrial activity, as defined in PCT Article 33(2) to (4) have been satisfied for all the claims presented in the application entering the national stage (37 CFR 1.492(a)(4)) \$96.00 <input type="checkbox"/> and the above requirements are not met (37 CFR 1.492(a)(1)) \$670.00 <input checked="" type="checkbox"/> U.S. PTO WAS NOT INTERNATIONAL PRELIMINARY EXAMINATION AUTHORITY Where no international preliminary examination fee as set forth in § 1.482 has been paid to the USPTO, and payment of an international search fee as set forth in § 1.445(a)(2) to the U.S. PTO: <input type="checkbox"/> has been paid (37 CFR 1.492(a)(2)) \$760.00 <input type="checkbox"/> has not been paid (37 CFR 1.492(a)(3)) \$970.00 <input checked="" type="checkbox"/> where a search report on the international application has been prepared by the European Patent Office or the Japanese Patent Office (37 CFR 1.492(a)(5))..... \$840.00				\$ 840.00
	Total of above Calculations				= \$2,450.00
SMALL ENTITY	Reduction by ½ for filing by small entity, if applicable. Affidavit must be filed. (note 37 CFR 1.9, 1.27, 1.28)				- \$
	Subtotal				\$2,450.00
	Total National Fee				\$2,450.00
	Fee for recording the enclosed assignment document \$40.00 (37 CFR 1.21(h)). (See Item 13 below). See attached "ASSIGNMENT COVER SHEET".				\$
TOTAL	Total Fees enclosed				\$2,450.00

- i. ☒ A check in the amount of \$2,450.00 to cover the above fees is enclosed.
 - ii. ☐ Please charge Account No. _____ in the amount of \$ _____.
- A duplicate copy of this sheet is enclosed.

**WARNING:

"To avoid abandonment of the application the applicant shall furnish to the United States Patent and Trademark Office not later than the expiration of 30 months from the priority date: * * * (2) the basic national fee (see § 1.492(a)) The 30-month time limit may not be extended." 37 C.F.R. § 1.495(b).

WARNING. *If the translation of the international application and/or the oath or declaration have not been submitted by the applicant within thirty (30) months from the priority date, such requirements may be met within a time period set by the Office. 37 C.F.R. § 1.495(b)(2) The payment of the surcharge set forth in § 1.492(e) is required as a condition for accepting the oath or declaration later than thirty (30) months after the priority date. The payment of the processing fee set forth in § 1.492(f) is required for acceptance of an English translation later than thirty (30) months after the priority date. Failure to comply with these requirements will result in abandonment of the application. The provisions of § 1.136 apply to the period which is set. Notice of Jan. 3, 1993, 1147 O.G. 29 to 40.*

3. ☒ A copy of the International application as filed (35 U.S.C. 371(c)(2)):

NOTE: *Section 1.495 (b) was amended to require that the basic national fee and a copy of the international application must be filed with the Office by 30 months from the priority date to avoid abandonment "The International Bureau normally provides the copy of the international application to the Office in accordance with PCT Article 20. At the same time, the International Bureau notifies applicant of the communication to the Office. In accordance with PCT Rule 47.1, that notice shall be accepted by all designated offices as conclusive evidence that the communication has duly taken place. Thus, if the applicant desires to enter the national stage, the applicant normally need only check to be sure the notice from the International Bureau has been received and then pay the basic national fee by 30 months from the priority date." Notice of Jan. 7, 1993, 1147 O.G. 29 to 40, at 35-36. See item 14c below*

- a. ☐ is transmitted herewith.
- b. ☐ is not required, as the application was filed with the United States Receiving Office.
- c. ☒ has been transmitted
 - i. ☒ by the International Bureau.
Date of mailing of the application (from form PCT/IB/308): 02/09/99.
 - ii. ☐ by applicant on _____
Date

4. ☒ A translation of the International application into the English language (35 U.S.C. 371(c)(2)):

- a. ☐ is transmitted herewith.
- b. ☒ is not required as the application was filed in English.
- c. ☐ was previously transmitted by applicant on _____
Date
- d. ☐ will follow.

5. ☒ Amendments to the claims of the International application under PCT Article 19 (35 U.S.C. 371(c)(3)):

NOTE: *The Notice of January 7, 1993 points out that 37 C.F.R. § 1.495(a) was amended to clarify the existing and continuing practice that PCT Article 19 amendments must be submitted by 30 months from the priority date and this deadline may not be extended. The Notice further advises that: "The failure to do so will not result in loss of the subject matter of the PCT Article 19 amendments. Applicant may submit that subject matter in a preliminary amendment filed under section 1.121. In many cases, filing an amendment under section 1.121 is preferable since grammatical or idiomatic errors may be corrected." 1147 O.G. 29-40, at 36.*

- a. ☐ are transmitted herewith.
- b. ☐ have been transmitted
 - i. ☐ by the International Bureau.
Date of mailing of the amendment (from form PCT/IB/308): _____.
 - ii. ☐ by applicant on _____
Date
- c. ☒ have not been transmitted as
 - i. ☒ applicant chose not to make amendments under PCT Article 19.
Date of mailing of Search Report (from form PCT/ISA/210): **06/07/99**.

- ii. ☐ the time limit for the submission of amendments has not yet expired. The amendments or a statement that amendments have not been made will be transmitted before the expiration of the time limit under PCT Rule 46.1.
6. ☒ A translation of the amendments to the claims under PCT Article 19 (38 U.S.C. 371(c)(3)):
- ☐ is transmitted herewith.
 - ☐ is not required as the amendments were made in the English language.
 - ☒ has not been transmitted for reasons indicated at point 5(c) above.
7. ☒ A copy of the international examination report (PCT/IPEA/409)
- ☒ is transmitted herewith.
 - ☐ is not required as the application was filed with the United States Receiving Office.
8. ☒ Annex(es) to the international preliminary examination report
- ☒ is/are transmitted herewith.
 - ☐ is/are not required as the application was filed with the United States Receiving Office.
9. ☒ A translation of the annexes to the international preliminary examination report
- ☐ is transmitted herewith.
 - ☒ is not required as the annexes are in the English language.
10. ☒ An oath or declaration of the inventor (35 U.S.C. 371(c)(4)) complying with 35 U.S.C. 115
- ☐ was previously submitted by applicant on _____.
Date
 - ☐ is submitted herewith, and such oath or declaration
 - ☐ is attached to the application.
 - ☐ identifies the application and any amendments under PCT Article 19 that were transmitted as stated in points 3(b) or 3(c) and 5(b); and states that they were reviewed by the inventor as required by 37 C.F.R. 1.70.
 - ☒ will follow.

Other document(s) or information included:

11. ☒ An International Search Report (PCT/ISA/210) or Declaration under PCT Article 17(2)(a):
- ☒ is transmitted herewith.
 - ☐ has been transmitted by the International Bureau.
Date of mailing (from form PCT/IB/308): _____.
 - ☐ is not required, as the application was searched by the United States International Searching Authority.
 - ☐ will be transmitted promptly upon request.
 - ☐ has been submitted by applicant on _____.
Date
12. ☒ An Information Disclosure Statement under 37 C.F.R. 1.97 and 1.98:
- ☒ is transmitted herewith.
Also transmitted herewith is/are:
☒ Form PTO-1449 (PTO/SB/08A and 08B).

- [X] Copies of citations listed.
- b. [] will be transmitted within THREE MONTHS of the date of submission of requirements under 35 U.S.C. 371(c).
- c. [] was previously submitted by applicant on _____
Date
13. [] An assignment document is transmitted herewith for recording.

A separate [] "COVER SHEET FOR ASSIGNMENT (DOCUMENT) ACCOMPANYING NEW PATENT APPLICATION" or [] FORM PTO 1595 is also attached.

14. [X] Additional documents:
- a. [X] Copy of request (PCT/RO/101)
- b. [] International Publication No. WO 99/41213
- i. [X] Specification, claims and drawing
- ii. [] Front page only
- c. [] Preliminary amendment (37 C.F.R. § 1.121)
- d. [X] Other

Form PCT/IPEA/408 (Written Opinion)

Response to Written Opinion dated 16 February 2000

Form PCT/IB/308, Form PCT/IB/332

15. [X] The above checked items are being transmitted
- a. [X] before 30 months from any claimed priority date.
- b. [] after 30 months.
16. [] Certain requirements under 35 U.S.C. 371 were previously submitted by the applicant on _____, namely:
- _____
- _____

AUTHORIZATION TO CHARGE ADDITIONAL FEES

WARNING: *Accurately count claims, especially multiple dependent claims, to avoid unexpected high charges if extra claims are authorized.*

NOTE: *"A written request may be submitted in an application that is an authorization to treat any concurrent or future reply, requiring a petition for an extension of time under this paragraph for its timely submission, as incorporating a petition for extension of time for the appropriate length of time. An authorization to charge all required fees, fees under § 1.17, or all required extension of time fees will be treated as a constructive petition for an extension of time in any concurrent or future reply requiring a petition for an extension of time under this paragraph for its timely submission. Submission of the fee set forth in § 1.17(a) will also be treated as a constructive petition for an extension of time in any concurrent reply requiring a petition for an extension of time under this paragraph for its timely submission." 37 C.F.R. § 1.136(a)(3).*

NOTE: *"Amounts of twenty-five dollars or less will not be returned unless specifically requested within a reasonable time, nor will the payer be notified of such amounts; amounts over twenty-five dollars may be returned by check or, if requested, by credit to a deposit account." 37 C.F.R. § 1.26(a).*

- [X] The Commissioner is hereby authorized to charge the following additional fees that may be required by this paper and during the entire pendency of this application to Account No. 04-1105.
- [X] 37 C.F.R. 1.492(a)(1), (2), (3), and (4) (filing fees)

WARNING:

Because failure to pay the national fee within 30 months without extension (37 C.F.R. § 1.492(b)(2)) results in abandonment of the application, it would be best to always check the above box.

430 Rec'd PCT/PTO 2 5 AUG 2000

☒ 37 C.F.R. 1.492(b), (c) and (d) (presentation of extra claims)

NOTE: Because additional fees for excess or multiple dependent claims not paid on filing or on later presentation must only be paid or these claims cancelled by amendment prior to the expiration of the time period set for response by the PTO in any notice of fee deficiency (37 C.F.R. § 1.492(d)), it might be best not to authorize the PTO to charge additional claim fees, except possible when dealing with amendments after final action.

☒ 37 C.F.R. 1.17 (application processing fees)

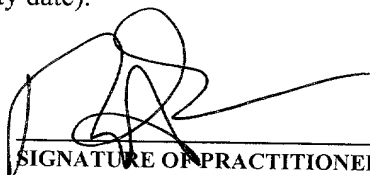
☒ 37 C.F.R. 1.17(a)(1)-(5)(extension fees pursuant to § 1.136(a).

☐ 37 C.F.R. 1.18 (issue fee at or before mailing of Notice of Allowance, pursuant to 37 C.F.R. 1.311(b))

NOTE: Where an authorization to charge the issue fee to a deposit account has been filed before the mailing of a Notice of Allowance, the issue fee will be automatically charged to the deposit account at the time of mailing the notice of allowance. 37 C.F.R. § 1.311(b).

NOTE: 37 C.F.R. 1.28(b) requires "Notification of any change in loss of entitlement to small entity status must be filed in the application . . . prior to paying, or at the time of paying . . . issue fee ." From the wording of 37 C.F.R. § 1.28(b): (a) notification of change of status must be made even if the fee is paid as "other than a small entity" and (b) no notification is required if the change is to another small entity

☐ 37 C.F.R. § 1.492(e) and (f) (surcharge fees for filing the declaration and/or filing an English translation of an International Application later than 30 months after the priority date).



SIGNATURE OF PRACTITIONER

Reg. No.: 33,860

Peter F. Corless

(type or print name of practitioner)

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Boston, MA 02109

Practitioner's Docket No. 55043

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: M. O'Brien et al.
Serial No.: Not Yet Assigned Group No.: Not Yet Assigned
Filed on: Herewith Examiner: Not Yet Assigned
For: HLA LINKED PRE-ECLAMPSIA AND MISCARRIAGE SUSCEPTIBILITY GENE

STATEMENT CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(f) and 1.27(b))--NONPROFIT ORGANIZATION

I hereby state that I am an official empowered to act on behalf of the nonprofit organization identified below:

Name of Nonprofit Organization National University of Ireland, Cork
Address of Nonprofit Organization College Road, Cork, Ireland

TYPE OF NONPROFIT ORGANIZATION

- ☒ University or Other Institution of Higher Education
☐ Tax Exempt Under Internal Revenue Service Code (26 USC 501(a) and 501 (c)(3))
☐ Nonprofit Scientific or Educational Under Statute of State of the United States of America
(Name of State _____
(Citation of Statute _____
☐ Would Qualify as Tax Exempt Under Internal Revenue Service Code (26 USC 501(a) and 501(c)(3)), if Located in the United States of America
☐ Would Qualify as Nonprofit Scientific or Educational Under Statute of State of the United States of America if Located in the United States of America
(Name of State _____
(Citation of Statute _____

I hereby state that the nonprofit organization identified above qualifies as a nonprofit organization, as defined in 37 CFR 1.9(e), for purposes of paying reduced fees to the United States Patent and Trademark Office under Sections 41(a) and (b) of Title 35, United States Code, with regard to the invention described in

- ☐ the specification filed herewith, with title as listed above.
☒ the application identified above.
☐ the patent identified above.

I hereby state that rights under contract or law have been conveyed to, and remain with, the nonprofit organization, with regard to the above identified invention.

If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37 C.F.R. 1.9(c), if that person made the invention, or by any concern that would not qualify as a small business concern under 37 CFR 1.9(d), or a nonprofit organization under 37 CFR 1.9(e)

*NOTE: Separate statements are required from each named person, concern or organization having rights to the invention as to their status as small entities (37 CFR 1.27).

Each such person, concern or organization having any rights in the invention is listed below:

- ☒ No such person, concern, or organization exists.
☐ Each such person, concern or organization is listed below.

Name _____
Address _____

☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

Name _____
Address _____

☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

(check the following item, if desired)

NOTE: The following verification statement need not be made in accordance with the rules published on October 10, 1997, 62 Fed. Reg. 52131, effective December 1, 1997

NOTE: "The presentation to the Office (whether by signing, filing, submitting, or later advocating) of any paper by a party, whether a practitioner or non-practitioner, constitutes a certification under § 10.18(b) of this chapter. Violations of § 10.18(b)(2) of this chapter by a party, whether a practitioner or non-practitioner, may result in the imposition of sanctions under § 10.18(c) of this chapter. Any practitioner violating § 10.18(b) may also be subject to disciplinary action. See §§ 10.18(d) and 10.23(c)(15)." 37 CFR 1.4(d)(2).

- ☐ I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Name of Person Signing _____
Title in Organization _____
Address of Person Signing National University of Ireland, Cork
College Road, Cork, Ireland

SIGNATURE 

Date 16/11/2000

HLA linked pre-eclampsia and miscarriage susceptibility gene

The present invention relates to a susceptibility gene for pre-eclampsia and eclampsia, and the use of such a gene in methods for diagnosing susceptibility to these diseases. The invention also relates to a test kit for diagnosis of susceptibility and to pharmaceutical compositions for the prevention or treatment of the diseases. The invention can also be used in the diagnosis of susceptibility to miscarriage and/or miscarriage-related infertility and/or intrauterine growth retardation.

Specifically, the present invention relates to methods and materials used to detect a HLA linked human pre-eclampsia and miscarriage predisposing gene (HLA-G), some alleles of which, or linked alleles of linked genes of which, cause susceptibility to pre-eclampsia and miscarriage. More specifically, the invention relates to sequence variation in the HLA-G gene and linked genes and their use in the diagnosis of susceptibility to pre-eclampsia and miscarriage. The invention further relates to sequence variations in the HLA-G gene and their use in diagnosis and prognosis of pre-eclampsia and miscarriage. Additionally, the invention relates to the therapy for pre-eclampsia and miscarriage and for susceptibility to pre-eclampsia and miscarriage including protein therapy, gene therapy and protein mimetics. The invention also relates to screening for drugs for pre-eclampsia and miscarriage therapy and for susceptibility to pre-eclampsia and miscarriage therapy. Finally, the invention relates to the screening of the HLA-G gene and linked genes for sequence variations which are useful for diagnosing susceptibility to pre-eclampsia and miscarriage.

Pre-eclampsia is the major cause of foetal and maternal morbidity and mortality with probable long term adverse effects on health due to the prolonged associated intrauterine hypoxia. Pre-eclampsia occurs in approximately five to ten percent of all population births and is uniquely a disease of pregnancy. Acute pathological changes begin to resolve soon after delivery. The pathologic mechanisms causing pre-eclampsia are unclear and no marker predictive for the disease prior to clinical evidence of the disease has been identified. Furthermore an association has been observed between miscarriage and pre-eclampsia (Cooper *et al.*, 1988).

Epidemiological studies show the disease to be highly heritable, mainly confined to first pregnancies and largely prevented by normal first pregnancy by the same partner. Patients affected in first pregnancies have a 13.1% recurrence risk for their second, whereas with a normal first pregnancy, the incidence in the second is of the order of 1%. Thus, the first pregnancy appears to have a significant protective effect against pre-eclampsia in a subsequent pregnancy. Therefore, it follows that pre-eclampsia is preventable in principle (Lie *et al.*, 1998).

Several classification schemes have been proposed to aid clinical recognition of pre-eclampsia. The classification advocated by the US National Institutes of Health working group on hypertension in pregnancy, is a rise in blood pressure of >15mm Hg diastolic or >30mm Hg systolic from measurement in early pregnancy, or to >140/90 mm Hg in late pregnancy if no early reading is available; plus proteinuria (>0.3g per 24 h) and/or odema. However, in practice, proteinuria measurements may not always be determined and symptoms additional to a rise in blood pressure such as headache, visual disturbance and/or epigastric pain indicate a deterioration in pregnancy consistent with pre-eclampsia and form a basis for clinical intervention of early delivery by caesarean section to resolve the condition. Spinillo *et al.* (1994) reported that women with pre-eclampsia had a significantly increased incidence of intrauterine growth retardation (IUGR) small for gestational age (SGA) infants.

Although the cause of pre-eclampsia is unknown, hypertension is observed in pre-eclampsia and has been the focus of a large amount of research on the disorder. However, the pathological and physiological changes of pre-eclampsia show that this syndrome is much more than pregnancy-induced hypertension. Evidence to date implicates the action of placental trophoblasts as the underlying cause.

In pre-eclampsia, cytotrophoblast invasion is shallow and spiral arteriol invasion is abnormal, resulting in reduced blood perfusion of the intervillous space. Moreover the characteristic pattern of integrin switching that takes place during normal trophoblast differentiation does not occur in pre-eclampsia.

The outermost layer (trophoblasts) of the human placenta is devoid of classical class I human leukocyte antigens (HLA-A and HLA-B) and class II proteins (HLA-DR, HLA-DQ and HLA-DP). Although this prevents recognition by maternal T lymphocytes, the lack of class I molecules leaves these cells susceptible to attack by natural killer (NK) cells. However, trophoblast cells directly in contact with maternal tissues selectively express a characteristic nonclassical class Ib molecule, HLA-G. HLA-E and limited HLA-C expression also occurs. Expression of HLA-G has been shown to be sufficient to protect otherwise susceptible target cells from NK cell mediated lysis. NK cells usually express several different inhibitory receptors of various specificities at the same time. Cross linking of any single inhibitory receptor is sufficient to inactivate NK cell activity against all possible targets. It has been shown that membrane bound HLA-G molecules were able to inhibit alloreactive NK cells with NK inhibitory receptor 1 and inhibitory receptor 2 (NK1 and NK2). It has been shown that CD94 / NKG2 is the predominant inhibitory receptor involved in recognition of HLA-G by decidual and peripheral NK cells. Thus, at a functional level, HLA-G is able to protect target cells from destruction by NK1-,NK2-and NKG2 specific effector cells (Loke and King, 1997). More recently, HLA-G has been shown to modulate the ability of blood mononuclear cells to release cytokines (Macjima *et al.* 1997) suggesting a role for HLA-G in triggering maternal-foetal immune interplay. Specifically, coculturing of HLA-G expressing cells with

peripheral blood mononuclear cells (PBMC) increased the amount of interleukin-3 (IL-3) and interleukin-1 beta (IL-1 beta) and decreased the amount of tumour necrosis factor-alpha (TNF-alpha) release from the PBMC cells.

HLA-G binds a diverse but limited array of peptides in a manner similar to that found for classical class I molecules and it has been reported that HLA-G is expressed in the human thymus raising the possibility that maternal unresponsiveness to HLA-G expressing foetal tissues may be shaped in the thymus by central presentation of this MHC molecule on the medullary epithelium (Crisa *et al.* 1997) HLA-G is known to be capable of stimulating a HLA-G restricted cytotoxic T lymphocyte response and HLA-G molecules can serve as target molecules in lytic reaction with cytotoxic T lymphocytes and HLA-G expressed internally in vivo in transgenic animals is involved in education of the lymphocytic repertoire (Schmidt *et al.*, 1997).

Major histocompatibility (MHC) molecules bind a diverse array of peptides for presentation to T cells as part of a mechanism for recognition of self and non-self cells and pathologically altered cells. A detailed analysis of peptides bound to the soluble and membrane HLA-G proteins shows that, like MHC class I molecules, HLA-G also binds a diverse, although less complex array of peptides (Lee *et al.*, 1995). Some of these peptides, which are derived from intracellular proteins, constitute minor histocompatibility antigens which in conjunction with MHC molecules provoke an immune reaction by blood mononuclear cells such as T cells. HLA bound peptides can readily be fractionated, fully or partially purified and sequenced and can be assayed for their capacity to promote an immune reaction by measurement of their capacity to reconstitute lysis of target cells by cytotoxic T cells (den Haan *et al.*, 1998).

The entire gene sequence of HLA-G is known and DNA sequence analysis of HLA-G has shown that the HLA-G gene exhibits limited polymorphism. van der Van & Ober, 1995 examined the first six exons of HLA-G in 45 healthy African-Americans and observed variations in exons 2 and 3, which correspond to the alpha 1 and alpha 2 domains of the peptide binding groove. The most common polymorphism observed was a C to T transition at position 1488, corresponding to codon 93. Another common polymorphism was identified by Harrison *et al.*, 1993 and is a 14 bp deletion in exon 8 of the gene. These results indicate that HLA-G is a polymorphic gene potentially capable of presenting a wide variety of peptides. Patterns of variability in HLA-G are similar to those of other class I MHC genes, where amino acid substitutions are clustered in the alpha 1 and alpha 2 domains.

Three observations of altered expression of HLA-G in pre-eclampsia have been reported. Colbern *et al.*, 1994 showed that the level of HLA-G in placental tissue was reduced in pre-eclampsia and that the decreased expression appeared to be related to a reduced number of trophoblasts in pre-eclamptic placental tissue. Hara *et al.*, 1996, showed that clusters of extravillous trophoblasts were devoid of HLA-

G in pre-eclamptic patients. Examination of human preimplantation blastocysts showed that only 40% of the blastocysts expressed HLA-G (Jurisicova, *et al.* 1996).

Inheritance

Several bodies of evidence show that pre-eclampsia and eclampsia are largely under genetic control.

5 However the genetic mechanisms underlying susceptibility to pre-eclampsia remain unclear. This is largely due to confounding factors peculiar to its inheritance. First, the condition is specific to pregnancy and genetic studies to date have not been able to clarify whether the genes responsible are acting through the maternal or foetal genotype or through some interaction between the two. Secondly, pre-eclampsia is largely confined to primagravidas with a much lower incidence in subsequent pregnancies and thirdly, as
10 the condition is specific to pregnancy, the genetic contribution of males is difficult to assess.

Diagnosis of true pre-eclampsia can be complicated by other hypertensive disorders such as essential hypertension and hypertension arising from renal disease. Such hypertensive disorders are distinct from true pre-eclampsia but nonetheless can confound diagnosis and thus pose problems for genetic studies.

The classification of pre-eclampsia by some investigators as a disease of immune dysfunction has
15 prompted a number of studies on the role of the major histocompatibility complex in the genetics of pre-eclampsia.

There are numerous published studies on HLA associations with pre-eclampsia (Cooper *et al.*, 1993). Besides the fact that the positive associations are, with one exemption, not reproduced in studies by others, these studies suffer from other difficulties. The number of individuals are generally small in
20 comparison to the large number of antigens at each of the HLA loci. There is a tendency for only significant associations to be reported and so there may be completed studies showing no association that have not been published besides those reported here. The first four associations reported are with antigen sharing or homozygosity. The number of antigens recognised has vastly increased with time. Antigens have been split as new sera become available, and the use of DNA techniques has split these further so
25 that there are over 100 HLA-A and 100 HLA-B alleles and over 25 HLA-DRB alleles (including five different, common sequences recognised as DR4 serologically). Thus what were typed as the same allele in homozygotes or shared antigens in the early studies cannot be relied on to be homogenous in sequence or function. Detecting homozygotes with sera in early studies suffers from the extra difficulty of distinguishing them from heterozygotes for another allele for which sera did not exist (blanks).

30 At least three studies have further investigated the association between pre-eclampsia and HLA-DR by linkage analysis (Winton *et al.*, 1990; Hayward *et al.*, 1992; Harrison *et al.*, 1997). In these definitive studies no evidence was found for linkage of the HLA region to pre-eclampsia. Hayward *et al.* (1992)

also investigated several candidate genes and random DNA markers. Overall, no evidence was found for linkage to several candidate genes implicated in the pathogenesis of hypertension and their results excluded linkage to several markers. In these studies, an autosomal recessive model was assumed. Winton *et al.* (1990) also analysed their data for a HLA linkage using the affected sib pair method and the affected pedigree-member method. Both of these methods make no assumption about the mode of inheritance and neither gave any indication of linkage. The majority of pre-eclampsia cases are considered sporadic. A familial pregnancy-induced hypertensive disorder has been described and two loci have been implicated in the familial form of the disorder, namely, a candidate region on chromosome 4 and the eNOS gene region on chromosome 7 (Harrison *et al.*, 1997, Arngrimsson *et al.*, 1997). The epidemiology of PET is consistent with familial pregnancy-induced hypertensive disorder and sporadic PET being distinct entities

Humphrey *et al.*, 1995, investigated the HLA-G deletion polymorphism for association with pre-eclampsia. Specifically, pre-eclamptic patients, offspring of pre-eclamptic mothers, blood relatives of pre-eclamptic patients, husbands of pre-eclamptic patients and a normal control group were genotyped for the polymorphism. There was no detectable association between pre-eclampsia in mothers or in offspring of pre-eclamptic mothers and the HLA-G deletion polymorphisms.

Karhukorpi *et al.*, 1997 investigated HLA-G polymorphisms for association with recurrent spontaneous miscarriage. Specifically, they showed that there was no association between several HLA-G restriction fragment length polymorphisms and recurrent spontaneous miscarriage.

In the largest study of monozygotic twins, pre-eclampsia was reported in five first pregnancies, and all affected mothers were discordant with their twin. A second well documented report on an identical set of twins also showed clear discordance for pre-eclampsia in their first pregnancies. These observations argue against a recessive model and further support a role for the foetal paternal genotype in the disorder. Furthermore, although the subject of some controversy, pre-eclampsia occurs in mothers with mono- and di-zygotic twins arguing against a recessive foetal genotype and in favour of a dominant paternal gene in the foetus.

Some studies have considered the possibility of changing paternity as a contributing factor in the occurrence of pre-eclampsia in multiparae. Most notably, a strong association between pre-eclampsia and changing paternity has been observed (Lie *et al.*, 1998).

Much of the work on pre-eclampsia has been based on the hypothesis of a major susceptibility locus in the affected mother and almost all of the genetic studies to date have focused on linkage or association between the genotype of the mother and pre-eclampsia. In order to test the hypothesis that foetal HLA-G

is the most likely candidate gene for the disorder, we have investigated HLA-G genotypes in pre-eclamptic and control trios and have shown that HLA-G is linked to both normal and pre-eclampsia pregnancy outcome and associated with recurrent spontaneous abortion. We have also investigated HLA-G genotypes in second pregnancies of control and pre-eclamptic trios and have shown that the presence of specific HLA-G alleles in the foetus in first pregnancy permits the occurrence of different HLA-G alleles in second pregnancy showing that HLA-G can induce tolerance to antigens in the first pregnancy and/or can modify the maternal immune system to accept foetuses in the second pregnancy in the absence of pregnancy related disorders that are selected against and/or cause pregnancy related disorders in first pregnancy.

Early pregnancy loss is the most common complication of human gestation of women attempting pregnancy. The majority of these losses are clinically unrecognised. Using a highly sensitive assay, the total incidence of miscarriage was estimated to be 31%, including 22% of losses which occurred at the very early stages of pregnancy i.e. before the pregnancy was clinically recognised (Wilcox *et al.*, 1988). Recurrent spontaneous abortion (RSA) or recurrent miscarriage, defined as the loss of three or more spontaneous pregnancies before 20 weeks gestation, occurs in less than 1% of pregnant women. Studies suggest that the chance of a successful pregnancy in an untreated woman who has experienced two or more first trimester miscarriages and no live births is approximately 30% to 50%. It is generally accepted that RSA is a condition with many different causes. however, about 50% of all RSA cases are not explained by structural genetic, endocrine, infectious or anatomic factors. Within the past few years there has been a growing recognition that recurrent pregnancy loss may have autoimmune (immunity against self) and alloimmune (immunity against another person) causes, even in women with no clinically diagnosed autoimmune diseases. This has lead to investigation of the role of the HLA system and RSA. in particular, much emphasis has been placed on the degree of sharing of HLA alleles and haplotypes between RSA couples. It has been suggested that fetuses whose HLA alleles do not differ from maternal alleles (i.e. histocompatible fetuses) are more likely to be aborted than fetuses with HLA alleles that differ from maternal alleles (i.e. histoincompatible fetuses). It would follow then that couples who match for HLA alleles or haplotypes would produce histocompatible fetuses and hence be at risk of miscarriage.

Ober *et al.* (1998) conducted a 10 year prospective study of HLA matching and pregnancy outcome. A significant increase in fetal loss was observed in couples who matched for a 16-locus haplotype encompassing the entire HLA locus. Christiansen *et al.* (1997) examined HLA-C and HLA-Bw in unexplained RSA couples. They found no variation in HLA-C, but a significantly higher number of RSA couples have the HLA-Bw4 haplotype than control couples. Jin *et al.*, (1995) examined the degree of sharing of HLA-A, HLA-B, HLA-DR and HLA-DQ haplotypes. They found a significant excess of

HLA-DR sharing in couples with RSA, and also a significant excess of HLA-DQ sharing in couples with unexplained infertility.

Several groups have recorded conflicting results. Billingham *et al.*, (1995) examined sharing of HLA-A, HLA-B, and HLA-DR alleles and found no higher degree of HLA sharing in couples with RSA than in fertile couples. Caudle *et al.*, (1983) reported similar findings. HLA-A, HLA-B and HLA-DR alleles were typed in a large population of unexplained RSA couples and in control couples (Sbracia *et al.*, 1996). No increased sharing in HLA alleles was observed. In addition, there was no difference in the frequency of HLA alleles between RSA couples and control couples. Saski *et al.*, (1997) reported an increase in the frequency of the HLA-DR4 allele in women who suffered from RSA compared to control women

The role of HLA sharing as a risk factor for RSA remains controversial, and no studies have reported any diagnostic or prognostic significance to HLA sharing in individual couples. In addition, reports of significant sharing of class II genes are difficult to explain as fetal cells in contact with the maternal immune system during pregnancy are devoid of HLA class II expression.

According to the present invention there is provided a method for diagnosing susceptibility to normal pregnancy, pre-eclampsia and/or eclampsia and/or intrauterine growth retardation and/or susceptibility to miscarriage and/or miscarriage-related infertility comprising the steps of:

- a) obtaining a fluid and/or tissue sample from a female and/or male and/or foetus; and either
- b) determining the sequence of all or part of the HLA-G nucleic acid, and/or HLA-G linked nucleic acid; or
- c) detecting variant forms of all or part of the HLA-G protein, and/or proteins encoded by HLA-G linked genes or:
- d) measuring the functional activity of all or part of the HLA-G encoding protein and/or proteins encoded by HLA-G linked genes or:
- e) measuring the size and/or level of all or part of HLA-G mRNA or mRNA transcribed from HLA-G linked genes or:
- f) measuring the size and/or level of all or part of HLA-G protein and/or protein encoded by HLA-G linked genes or:
- g) quantifying cells or molecules whose concentration changes as a result of HLA-G action; and
- h) comparing any of the parameters b) to g) with those of a female and/or male and/or foetus of a normal pregnancy and/or a pregnancy with pre-eclampsia and/or eclampsia and/or intrauterine growth retardation and/or susceptibility to miscarriage and/or miscarriage-related fertility outcome.

Preferably the HLA-G nucleic acid is analysed by the presence of the C and/or T allele of codon 93 in exon 3 and/or the insertion and/or deletion allele of exon 8.

Preferably the effect of one or more of the HLA-G sequence variants on the functional activity of HLA-G and/or on the size and/or the level of all or part of the HLA-G mRNA and/or its encoded peptide is measured.

In its simplest the present invention provides a method of diagnosing susceptibility to pre-eclampsia and/or eclampsia and/or intrauterine growth retardation and/or susceptibility to miscarriage and/or miscarriage-related infertility comprising the steps of:

- a) obtaining nucleic acid from a parent and/or a prospective parent and/or foetus;
- b) establishing the HLA-G sequence variants present in the parent and/or foetus by analysing the nucleic acid isolated in step (a); and
- c) comparing the HLA-G sequence variants identified in step (b) with known HLA-G sequence variants.

Preferably, the HLA-G sequence variants are established by characterising all or part of the DNA sequence of the HLA-G gene by methods selected from DNA sequencing, PCR-restriction fragment length polymorphism analysis, glycosylase mediated polymorphism detection, oligonucleotide hybridisation, gel electrophoretic detection of polymorphisms and amplification based detection approaches.

Suitably, a stratified approach is used whereby the C/T-93 in exon 3 and insertion/deletion polymorphism in exon 8 are first genotyped, followed by genotyping of other variations in exon 3, exon 2, intron 2, followed by exon 1 and 4, followed by the remainder of the HLA-G gene.

The invention also provides a test kit for the diagnosis of susceptibility to normal pregnancy, pre-eclampsia and/or eclampsia and/or intrauterine growth retardation and/or susceptibility to miscarriage and/or miscarriage-related infertility comprising:

- a) oligonucleotide primers for amplification of all or part of the HLA-G gene and/or HLA-G linked DNA;
- b) amplification reagents for amplification of genomic DNA and/or RNA segments, selected from a DNA / RNA polymerase, a reverse transcriptase, the deoxyribonucleotides dATP, dCTP, dGTP, dTTP and dUTP, and/or ribonucleotides ATP, CTP, GTP, TTP and UTP, and reaction buffer;
- c) reagents for identifying sequence variants in DNA and/or RNA;
- d) control DNA and/or RNA.

Preferably the primers of (a) allow specific amplification of all or part of the HLA-G gene using the polymerase chain reaction. Several polymorphisms are known to occur in the HLA-G gene. A C to T

polymorphism occurs at nucleotide 1488 in the third position of codon 93 and is referred to as C/T-93 herein where C-93 is one allele of the polymorphism and T-93 is the other allele of the polymorphism. Suitably, the C/T-93 polymorphism is genotyped by PCR amplification of a section of intron 2 - exon 3 using the primers 5'-TACTCCCGAGTCTCCGGGTCTG-3' (SEQ ID NO. 1) as the forward primer and 5'-AGGCGCCCCACTGCCCCTGGTAC-3' (SEQ ID NO. 2) as the reverse primer giving rise to the amplified C-93 allele (SEQ ID NO. 4) and amplified T-93 allele (SEQ ID NO. 5) followed by semi-nested PCR amplification using the forward primer 5'-GACCGAGGGGGTGGGGCCAGGTTCT-3' (SEQ ID NO. 3) and the reverse primer 5'-AGGCGCCCCACTGCCCCTGGTAC-3' (SEQ ID NO. 1). In the semi-nested amplification reaction dTTP is replaced by dUTP. The 3' end of the forward primer is designed so that the first U incorporated downstream of the forward primer is at, or distal to, the polymorphic site in codon 93. Following amplification using end labelled forward primer, glycosylase mediated cleavage of the amplified product is performed. Cleavage products are resolved by denaturing gel electrophoresis (20% polyacrylamide) and visualised by autoradiography. The C-93 allele is detected as a 32 n fragment (SEQ ID NO. 8) and the T-93 allele as a 27 n fragment (SEQ ID NO. 9).

The common 14 base pair insertion / deletion polymorphism in exon 8 of the HLA-G gene is referred to as I/D-E8 herein where I-E8 is one allele of the polymorphism and D-E8 is the other allele of the polymorphism. Suitably, genotyping of the HLA-G exon 8 deletion polymorphism is performed by amplifying a short section flanking the deletion location in exon 8. This is achieved using the polymerase chain reaction with primers designed to hybridise to known DNA sequence in exon 8. The forward primer is 5'-TGTGAAACAGCTGCCCTGTGT-3' (SEQ ID NO. 10) and the reverse primer is 5'-AAGGAATGCAGTTCAGCATGA-3' (SEQ ID NO. 11). The I/D exon 8 polymorphism is genotyped by size separation of the PCR products on a 10% non denaturing polyacrylamide gel and visualised by staining with ethidium bromide. the I-E8 insertion allele giving rise to a 151 bp product (SEQ ID NO. 12) and the D-E8 deletion allele giving rise to a 137 bp product (SEQ ID NO. 13).

Suitably, allele specific genotyping is performed in cases where maternal and paternal C/T-93 and I/D-E8 HLA-G haplotypes cannot be directly assigned. This is achieved using allele specific primers which allows selective amplification of the I-E8 or D-E8 allele. Following allele specific amplification, the C/T-93 polymorphism is then genotyped using the GMPD assay described above. Primers for amplification of the I-E8 allele are 5'-TACTCCCGAGTCTCCGGGTCTG-3' (SEQ ID NO. 1) as the forward primer and 5'-CAAAGGGAAGGCATGAACAAATCTTG-3' (SEQ ID NO. 14) as the reverse primer. Primers for amplification of the D-E8 allele are 5'-TACTCCCGAGTCTCCGGGTCTG-3' (SEQ ID NO. 1) as the forward primer and 5'-GTTCTTGAAGTCACAAAGGGAAGT -3' (SEQ ID NO. 15) as the reverse primer. Such allele specific amplification gives rise to four possible haplotypes, namely I-E8 and C-93

haplotype (SEQ ID NO. 16), I-E8 and T-93 haplotype (SEQ ID NO. 17), D-E8 and C-93 haplotype (SEQ ID NO. 18) and D-E8 and T-93 haplotype (SEQ ID NO. 19).

Suitably, haplotypes are constructed and suitably, transmitted and non-transmitted alleles to offspring /
5 foetus are assigned.

Preferably, the amplification reagents include a thermostable DNA polymerase, amplification buffer and DNA precursor nucleotides.

All or part of any HLA-G sequence and/or HLA-G linked sequence may also be amplified, by a method
10 or combination of methods selected from nucleic acid sequence based amplification, self-sustained sequence replication, transcription-mediated amplification, strand displacement amplification and the ligase chain reaction.

Preferably the comparison of one or more variants identified is performed by association and/or linkage analysis and/or transmission analysis. Preferably all or part of the HLA-G sequence is cloned into a vector.

15 The invention may involve:

- a) obtaining nucleic acid or fluid or tissue sample from a parent and/or prospective parent and/or foetus;
- b) establishing the HLA genotype or serotype of the parent/prospective parent and/or foetus by analysing the nucleic acid or fluid or tissue sample isolated in step (a);
- 20 c) comparing the HLA genotypes or serotypes identified in step (b) with known HLA genotypes or serotypes respectively.

Preferably the method involves the measuring of cellular and/or soluble HLA-G levels. Preferably HLA-G levels are measured by immunoassay using an antibody for specific HLA-G protein.

The invention may involve identifying the variant form of HLA-G protein and/or the levels thereof present
25 in the sample. Preferably HLA-G variant proteins and/or levels thereof are detected and/or quantified by immunoassay using specific antibodies which detect HLA-G variants, or HLA-G protein. Alternatively, antibody specific for HLA-G protein variants and/or electrophoretic separation methods and/or chromatographic separation methods may be used. Preferred methods for detecting HLA-G protein and variants thereof include, enzyme linked immunosorbent assays (ELISA), radioimmuno-assays (RIA),
30 immunoradiometric assays (IRMA) and immunoenzymatic assays (IEMA), including sandwich assays using monoclonal and/or polyclonal antibodies.

The invention may involve measuring of level of molecules whose concentration changes as a direct and/or indirect result of HLA-G action. Preferably the molecules are selected from IL-1, IL-2, IL-3, IL-4, IL-6, IL-10 beta and tumour necrosis factor alpha. Preferably the levels of such molecules are measured by immunoassay using antibodies specific for the molecules.

Alternatively, the method may involve measuring of levels of trophoblast specific markers. Preferably the trophoblast markers are cytokeratins pregnancy specific glycoprotein 1, human chorionic gonadotrophin and human placental lactogen. Preferably the levels of such molecules are measured by immunoassay using antibodies specific for the molecules.

In one embodiment the method may comprise the steps of:

- a) incubating blood mononuclear cells and/or a subset of such cells with one or more HLA-G variants and/or any combination thereof and/or cells expressing all or part of one or more variants of the HLA-G gene and/or a combination of one or more variants thereof, wherein the blood mononuclear cells and/or HLA-G variant is from a female and/or male and/or foetus
- b) analysing the activity of the blood mononuclear cells and/or the HLA-G and/or cells expressing one or more HLA-G variant.

Preferably, the blood mononuclear cells are obtained as a blood sample and/or tissue sample from the female and/or are obtained through matching the females blood mononuclear cells with blood mononuclear cells from a donor and/or cell line panel. Preferably populations of T cells and/or NK cells are isolated from the blood sample by density centrifugation and/or immunoselection. Preferably, blood mononuclear cells matching the females blood mononuclear cells are identified from a test panel by matching the HLA serotype and/or extended HLA genotype and/or HLA-G genotype of the female with the HLA serotype and/or extended HLA genotype and/or HLA-G genotype of blood mononuclear cells. Preferably, HLA-G matching the male and/or female HLA-G is identified from a test panel by matching the HLA-G type and/or HLA-G genotype of the male and/or female with the HLA-G type and/or HLA-G genotype of HLA-G proteins and/or cells expressing one or more HLA-G gene variants in the test panel. Preferably, such a test panel is assembled by growing cells expressing one or more HLA-G variants. Such cells may be derived from natural tissue such as placenta and/or created artificially by the introduction of one or more vectors bearing HLA-G gene variants which are capable of promoting the expression of the HLA-G gene into a cell and/or by inducing the expression of native HLA-G in cells. Suitably, the vector used is plasmid, phage, viral, and/or artificial chromosome based. Preferably HLA-G protein is used as a crude preparation and/or fully or partially purified from such cells. HLA-G protein may be loaded with binding peptides naturally or artificially.

Preferably, the HLA-G - blood mononuclear cell interaction is measured by assessing blood mononuclear cell activation including assessment of one or more of the following: cell proliferation, transformation cytotoxic response, surface marker expression, cytokine production, conjugate formation and target specificity.

5 The method may comprise the steps of:

- a) cloning the HLA-G gene from a parent and/or prospective parent and/or foetus;
 - b) expressing the HLA-G protein from the cloned gene *in vitro* and/or *in vivo*;
 - c) measuring the levels of activity of the expressed HLA-G protein;
 - d) comparing the levels of activity of the expressed HLA-G protein with the levels of activity observed
- 10 for the normal HLA-G protein.

The method may also comprise:

- a) establishing all or part of the HLA-G sequence and/or HLA-G linked sequences present in a sample from a female and/or male and/or foetus by analysing the nucleic acid from said sample;
 - b) determining whether one or more of any variants or any combination thereof, identified in step (a) are
- 15 indicative of susceptibility to normal pregnancy or pre-eclampsia and/or eclampsia and/or intrauterine growth retardation and/or susceptibility to miscarriage and/or miscarriage-related infertility by comparative analysis and/or analysis of the effect of one or more of the variants on the functional activity of HLA-G and/or on HLA-G mRNA.

Preferably, the HLA-G sequence variants are established by characterising all or part of the DNA sequence of the HLA-G gene and/or closely linked DNA including HLA-A, HLA-B, HLA-C, HLA-E, HLA-F and HLA-H genes by amplifying all or parts HLA-G or closely linked DNAs and identifying the sequence variants present using one or more sequence variation detection methods.

20

Suitably, one or more copies of all or parts of the HLA-G gene is amplified by any of several amplification approaches such as the polymerase chain reaction (PCR), nucleic acid sequence based amplification (NASBA), self sustained sequence replication (3SR), transcription-mediated amplification (TMA) and strand displacement amplification. Amplification of a target nucleic acid molecule may also be carried out using a the ligase chain reaction (LCR) and a variation of the LCR which employs a short PCR step (PLCR). Suitably, DNA or mRNA is used as the amplification substrate. Suitably, mRNA is converted into DNA using reverse transcriptase. Suitably, the amplified molecules are analysed directly

25 and/or may be cloned into a vector to facilitate analysis. Suitably, DNA sequence variations are detected by any one or more of a variety of gene variation detection methods including DNA sequencing, glycosylase mediated polymorphism detection, restriction fragment length polymorphism analysis, enzymatic or chemical cleavage assays, hybridisation to DNA probe arrays, allele specific oligonucleotide hybridisation assays, allele specific amplification methods such as the amplification refractory method

30

(ARMS), electrophoretic detection of polymorphisms based on migration through a gel matrix, 5' nuclease assay and ligase chain reaction.

Suitably, it can be determined if one or more variants identified are known variants associated with susceptibility to normal pregnancy and/ or susceptibility to pre-eclampsia and/or eclampsia and/or intrauterine growth retardation and/or susceptibility to miscarriage and/or miscarriage-related infertility. Alternatively, comparative analysis is performed by gene association and/or gene linkage methods to determine whether HLA-G variants and/or HLA-G linked variants are associated with normal pregnancy and/ or susceptibility to pre-eclampsia and/or eclampsia and/or intrauterine growth retardation and/or susceptibility to miscarriage and/or miscarriage-related infertility.

Alternatively, HLA-G variants associated with normal pregnancy and/ or susceptibility to pre-eclampsia and/or eclampsia and/or intrauterine growth retardation and/or susceptibility to miscarriage and/or miscarriage-related infertility can be identified by the effect of HLA-G variants on HLA-G function. Suitably, HLA-G variants are functionally analysed by measuring the interaction of one or more of the HLA-G variants and/or any combination thereof, with blood mononuclear cells and/or measuring the size and level of the HLA-G messenger RNA and/or the size and level of HLA-G gene product and/or peptide binding for one or more of the HLA-G variants and/or any combinations thereof. HLA-G - blood mononuclear cell activity is measured by assessing blood mononuclear cell activation including assessment of one or more of the following; cell proliferation, cytotoxic response, surface marker expression, cytokine production, conjugate formation and target specificity.

The invention also relates to a pharmaceutical composition comprising a pharmaceutically effective amount of HLA-G protein and/or cells expressing HLA-G and/or one or more peptides which binds to HLA-G and/or blood mononuclear cells from a donor and/or a cells from a test panel known to interact with HLA-G variants, cytokines and any combination thereof including IL-1 beta, IL-2, IL-3, IL-4, IL-6, IL-10 and tumour necrosis factor-alpha and/or inhibitors of cytokines and/or tumour necrosis factor alpha and/or derivatives of cytokines and/or tumour necrosis factor-alpha, optionally with pharmaceutically-acceptable carriers or excipients.

The invention also provides a method for screening for agents which can potentially be used as diagnostic indicators and/or drug targets for pre-eclampsia, miscarriage, miscarriage-related infertility and intrauterine growth retardation by:

a) measuring the expression level of one or more genes and / or proteins in HLA-G expressing cells and /or blood mononuclear cells and / or T cell and /or natural killer cell subsets thereof following interaction with HLA-G and / or HLA-G expressing cells;

b) comparing the expression level identified in step (a) with the expression level in HLA-G expressing cells and /or the blood mononuclear cells and / or T cell and /or natural killer cell subsets thereof following interaction with HLA-G and / or HLA-G expressing cells in normal pregnancy and/ or pre-eclampsia pregnancy and/or intrauterine growth retardation pregnancy and/or miscarriage pregnancy and/or miscarriage-related infertility.

In a further aspect the invention provides a method for screening for potential pre-eclampsia and eclampsia and intrauterine growth retardation and miscarriage and miscarriage-related infertility therapeutic agents selected from:

- a) identifying agents which alter the expression of HLA-G;
- b) identifying agents which alter the activity of HLA-G;
- c) identifying agents which mimic the action of HLA-G;
- d) identifying agents which bind to HLA-G;
- e) identifying peptides which bind to HLA-G;
- f) identifying agents which bind to HLA-G receptors;
- g) identifying expressed genes using DNA probe arrays in a cellular background in HLA-G expressing cells and/or blood mononuclear cells interacting with HLA-G and/or cells expressing HLA-G interacting with blood mononuclear cells;
- h) identifying expressed genes using DNA probe arrays in a cellular background whose expression is altered in response to HLA-G expression in the cells and/or in response to interacting cells expressing HLA-G;
- i) identifying expressed proteins using mass spectrometry methods in HLA-G expressing cells and/or blood mononuclear cells interacting with HLA-G and/or cells expressing HLA-G interacting with blood mononuclear cells.

Preferably sperm and/or semen and/or female reproductive tissue are screened for agents:

- a) which alter the expression of HLA-G in fertilised eggs and/or embryos;
- b) which alter the cell cleavage rate of fertilised eggs and/or embryos;
- c) which induce cellular factors in cell in culture and/or cell in vivo that alter the cell cleavage rate of fertilised eggs and/or embryos.

The method may involve:

- a) measuring the expression level of one or more genes and/or proteins in HLA-G expressing cells and/or blood mononuclear cells and/or T cell and/or natural killer cell subsets thereof following interaction with HLA-G and/or HLA-G expressing cells;
- b) comparing the expression level identified in step (a) with the expression level in HLA-G expressing cells and/or the blood mononuclear cells and/or T cell and/or natural killer cell subsets thereof following interaction with HLA-G and/or HLA-G expressing cells associated with normal pregnancy and/ or pre-

eclampsia pregnancy and/or intrauterine growth retardation pregnancy and/or miscarriage pregnancy and/or miscarriage-related infertility.

Preferably, blood mononuclear cells and/or HLA-G expressing cells are obtained from a female and/or male and/or foetus and/or test panel of blood mononuclear cells and/or HLA-G expressing cells.

- 5 Preferably, gene expression is measured by any one or combination of several methods including hybridisation between cDNA and/or RNA from the cells and DNA probes and/or RNA probes and/or DNA probe arrays, quantitative amplification approaches such as quantitative (reverse transcriptase - polymerase chain reaction) RT-PCR, 5' nuclease assay, ribonuclease protection assay and S1 nuclease assay.
- 10 Preferably, protein expression is measured by any one or combination of several methods including one dimensional and/or two dimensional gel electrophoresis and staining of proteins and/or detection of one or more proteins using, enzyme linked immunosorbent assays (ELISA), radioimmunoassays (RIA), immunoradiometric assays (IRMA) and immunoenzymatic assays (IEMA), including sandwich assays and Western blotting using monoclonal and/or polyclonal antibodies.

15 Alternatively, the method may involve:

- a) measuring the expression level of one or more genes and/or proteins in cells expressing HLA-G; and
- b) comparing the expression level identified in step (a) with the expression level in HLA-G non-expressing cells.

Preferably, the cells are fertilised animal eggs and/or animal embryos.

- 20 The invention also provides a method for the prevention of pre-eclampsia and/or eclampsia and/or intrauterine growth retardation and/or susceptibility to miscarriage and/or miscarriage-related infertility selected from:
 - a) treatment of a female with all or part of a pharmaceutically effective amount of an effective HLA-G protein and/or peptides which bind to HLA-G and/or cells expressing HLA-G;
 - 25 b) treatment of a female with all or part of a pharmaceutically effective amount of molecules or inhibitors of molecules whose level or activity is directly or indirectly altered by HLA-G;
 - c) treatment of a female with all or part of a pharmaceutically effective amount of an agent which alters HLA-G expression;
 - d) treatment of a female with all or part of a pharmaceutically effective amount of an agent which
 - 30 alters NK cell activity;
 - e) treatment of a female with all or part of a pharmaceutically effective amount of an agent which mimics all or part of HLA-G action;

- f) treatment of a female with all or part of a pharmaceutically effective amount of molecules which inhibit the interaction between HLA-G and one or more of its receptors;
- g) treatment of a female with all or part of a pharmaceutically effective amount of an agent which alters the size and/or level of HLA-G mRNA;
- h) treatment of a female with all or part of a pharmaceutically effective amount of an agent which alters HLA-G related blood mononuclear cell activity;
- i) treatment of a female with blood mononuclear cells that recognise foetal and/or self HLA-G;
- j) treatment of a female with HLA-G protein and/or cells expressing HLA-G.

The invention may comprise:

- a) obtaining blood mononuclear cells and/or T cell and/or natural killer cell subsets thereof and/or HLA-G and/or HLA-G expressing cells from a female and/or male and/or foetus and/or test panel;
- b) measuring the expression level of one or more genes and/or proteins in the HLA-G expressing cells and/or blood mononuclear cells following interaction with HLA-G and/or HLA-G expressing cells;
- c) comparing the expression level identified in step (b) with the expression level in the blood mononuclear cells and/or HLA-G expressing cells in normal pregnancy and/or pre-eclampsia pregnancy and/or intrauterine growth retardation pregnancy and/or miscarriage pregnancy and/or miscarriage-related infertility.

Preferably, the blood mononuclear cells and/or HLA-G expressing cells are obtained as a blood sample and/or tissue sample. Preferably populations of T cells and/or NK cells are isolated from the blood sample by density centrifugation and/or immunoselection. Preferably HLA-G expressing cells are isolated by immunoselection.

The invention also provides a method for improving fertility and pregnancy outcome wherein male and/or female partners and/or sperm and/or ova and/or recipients of fertilised eggs and/or zygotes / and/or embryos are selected on the basis of HLA-G so that their genotypes and/or serotypes are associated with normal pregnancy outcomes and/or not associated with pre-eclampsia and/or eclampsia and/or intrauterine growth retardation and/or susceptibility to miscarriage and/or miscarriage-related infertility.

In particular there is provided a method for improving pregnancy success selected from:

- a) pre-treating the female with sperm and/or attenuated forms thereof, and/or semen and/or fractions thereof from a male with a known HLA-G genotype, prior to mating with a male of a different HLA-G genotype, and/or in vitro fertilisation using sperm from a male of a different HLA-G genotype and/or embryo transfer where the male HLA-G is of a different HLA-G genotype;
- b) mixing sperm of a known HLA-G genotype with sperm and/or attenuated forms thereof, and/or semen and/or fractions thereof from a male with a different HLA-G genotype prior to in vitro fertilisation.

Preferably fertility and/or pregnancy outcome are improved by selection of male and / or female partners and / or sperm and / or ova and / or recipients of fertilised eggs and / or zygotes / and / or embryos so that (a) their HLA-G and /or HLA genotypes and /or serotypes or (b) the activity of their HLA-G and / or blood mononuclear cells interacting with HLA-G are indicative of normal pregnancy outcomes and / or not associated with pre-eclampsia and/or eclampsia and/or intrauterine growth retardation and/or susceptibility to miscarriage and/or miscarriage-related infertility.

Cloning of all or part of one or more HLA-G genes in any of the above methods may be achieved by amplification of all or part of one or more HLA-G genes and insertion of all or part of the amplified product into a vector capable of expressing the inserted gene. Expression of the HLA-G protein from the cloned gene in any of the above methods may be achieved by introduction of the expression vector into a suitable host such as a bacterium or an eukaryotic cell in culture. The level of activity of the expressed HLA-G protein in any of the above methods may be achieved by a) directly and/or indirectly measuring the interaction of the HLA-G protein and/or cells expressing HLA-G protein with blood mononuclear cells and/ or b) detecting one or more molecules whose level is altered as a result of the interaction of the HLA-G protein and/or cells expressing HLA-G protein with blood mononuclear cells and/or c) measuring changes in cell cleavage rate due to direct and/or indirect action of the HLA-G protein and/or cells expressing HLA-G protein with blood mononuclear cells.

HLA-G as defined herein refers to any form of HLA-G and / any complex involving HLA-G including different isoforms of HLA-G arising from alternative splicing pathways, combination of different HLA-G isoforms, secreted HLA-G, membrane bound HLA-G HLA-G with peptides bound and HLA-G associated with beta -2-microglobulin. HLA-G protein refers to any crude, partially and/or fully purified form of HLA-G.

The invention also provides use of a DNA sequence selected from any one of sequence I.D.s 1 to 21 for diagnosis of susceptibility to or in a test kit for the diagnosis of susceptibility to normal pregnancy, pre-eclampsia and/or eclampsia and/or intrauterine growth retardation and/or susceptibility to miscarriage and/or miscarriage-related infertility, for monitoring progress of pregnancy, for use in the manufacture of a medicament, in a method for screening potential therapeutic agents, in a method for screening for potential diagnostic indicators and/or drug targets, in a method for improving pregnancy success or in a method for the prevention of pre-eclampsia and/or eclampsia and/or intrauterine growth retardation and/or susceptibility to miscarriage and/or miscarriage-related infertility, for monitoring progress of pregnancy.

The invention also provides a method for induction of tolerance in a host to a non-self tissue which comprises administering HLA-G and /or HLA-G loaded with peptides from the non-self tissue and /or

HLA-G expressing cells derived from or related to the non-self tissue, and/or a non-self tissue bearing an introduced HLA-G gene so that HLA-G is expressed in all or part of the tissue.

In a further aspect the invention provides a method for the treatment of autoimmune disease which comprises administering HLA-G and /or HLA-G loaded with peptides from a self or non-self tissue and /
5 or with specific autoimmune antigen and /or HLA-G expressing cells from a self and/or non-self tissue and/or a self and/or non-self tissue bearing an introduced HLA-G gene so that HLA-G is expressed in all or part of the tissue.

Methods

Identification of Subjects

10 In the initial phase of sampling pre-eclamptic patients were identified as primagravidas who were delivered by caesarean section at or prior to 36 weeks gestation because of a deterioration in pregnancy indicative of pre-eclampsia. Diagnostic symptoms were a rise in blood pressure >15mm Hg diastolic or >30mm Hg systolic from measurement in early pregnancy or to >140/90mm Hg in late pregnancy, and one or more of the following: proteinuria, odema, headache, visual disturbance, epigastric pain. Control
15 patients were identified as primagravidas with normal delivery and normal blood pressure. 5-10mls of blood were taken from the offspring of primagravida pre-eclampsia and normal pregnancies with informed consent.

In the second phase of sampling, blood samples and/or cheek swab sample for DNA extraction were collected from control trios following delivery. The appropriate informed consent was obtained from all
20 subjects. Control mothers were identified as primagravidas under the age of thirty three with normal delivery and normal blood pressure. All individuals were Irish and Caucasian by origin. Mothers were interviewed to ensure that they were primagravidas. Primagravida (first pregnancy) pre-eclampsia trios where the mothers suffered severe pre-eclampsia and a matching control group of normal primagravida trios were identified and sampled. Families (mother, father, first and second offspring) where the mother
25 had two or more successful normal pregnancies in the absence of pregnancy related disorders including pre-eclampsia and miscarriage were also identified and sampled. Families (mother, father, first and second offspring) where the mother had pre-eclampsia in the first pregnancy and a normal second pregnancy in the absence of pregnancy related disorders including pre-eclampsia and miscarriage were also identified and sampled. Couples with recurrent spontaneous abortion were also identified and sampled. To minimise
30 the possibility of misdiagnosis of PE, we applied stringent criteria to ascertainment of samples. Essentially pre-eclampsia cases were identified as primagravidas under the age of 35 who were delivered by caesarean section at, or prior to, 36 weeks gestation because of a deterioration in pregnancy indicative

of pre-eclampsia. Diagnostic symptoms were a rise in blood pressure of >15mm Hg diastolic or >30mm Hg systolic from measurement in early pregnancy or to >140/90mm Hg in late pregnancy, and one or more of the following: proteinuria, odema, headache, visual disturbance, epigastric pain. Diagnostic symptoms were completely resolved within 3 months after delivery. A preliminary survey of the sisters of the pre-eclamptic women in this study did not reveal an increased incidence of the condition, indicating that pre-eclampsia in the cohort of mothers investigated here is sporadic. A cohort of couples where the mother had three or more consecutive miscarriages were identified (recurrent miscarriage).

Genotyping of HLA-G polymorphism

Genomic DNA was extracted from peripheral blood samples and/or cheek swab samples by standard methods. DNA concentration was determined by absorbance at 260nm for samples where DNA was isolated from blood. The integrity and purity of the genomic DNA was determined by agarose gel electrophoresis and OD260:OD280 ratio respectively.

The C-93T HLA-G polymorphism is also known as the C/T codon 93 polymorphism (and as HLA-G C1488T) and referred to as C/T-93 herein where C-93 is one allele of the polymorphism and T-93 is the other allele of the polymorphism. In order to genotype the C/T-93 polymorphism in the genomic DNA samples, exon 3 of the HLA-G gene was first amplified using the polymerase chain reaction with primers designed to hybridise to the known DNA sequence flanking exon 3. The forward primer was 5'-TACTCCCGAGTCTCCGGGTCTG-3' (SEQ ID NO. 1) and the reverse primer was 5'-GAGGCGCCCCACTGCCCTGGT-3'.

The polymerase chain reaction was carried out in a total volume of 25µl, with 100ng genomic DNA, 50ng of each primer, 0.2mM of each deoxynucleoside triphosphate (dATP, dCTP, dGTP and dTTP) 50mM KCl, 10mM Tris-HCl, pH 9.0 at 25°C, 0.1% Triton X-100, 1.5mM MgCl and 0.5U of Taq Polymerase. Reaction mixtures were covered with an equal volume of mineral oil and amplification was carried out using the "hot start" technique in a thermal cycler. The conditions for amplification involved denaturation at 94 °C for 5 min followed by addition of Taq Polymerase. Thirty cycles were then performed: 94 °C for 1 min, 63°C for 1 min, 72°C for 1 min and finally a 10 min extension at 72°C.

Genotyping of the C/T-93 HLA-G polymorphism was then performed using a semi nested amplification approach and the Glycosylase Mediated Polymorphism Detection method (Vaughan and McCarthy 1998). A 319bp section of the HLA-G gene encompassing the C/T-93 polymorphism location was amplified using a semi nested polymerase chain reaction approach from the previously amplified exon 3 of the HLA-G gene using the exon 3 reverse primer and the internal forward primer 5'-GACCGAGGGGGTGGGGCCAGGTTCT-3' (SEQ ID NO. 3). The forward primer was end labelled by

incubation with polynucleotide kinase in the manufacturers buffer (New England Biolabs) and 5µCi 32P-ATP (3000Ci/mmol) for 30 min at 37°C followed by ethanol precipitation to remove unused labelled nucleotide. The semi nested amplification reaction was carried out in a total volume of 10µl, with 1µl of a 1 in 500 dilution of the previously amplified exon 3 product, 3pmoles of forward and reverse primer, 0.2mM of each deoxynucleoside triphosphate (dATP, dCTP, dGTP and dUTP) 50mM KCl, 10mM Tris-HCl, pH 9.0 at 25°C, 0.1% Triton X-100, 1.5mM MgCl and 0.5U of Taq Polymerase. Reaction mixtures were covered with an equal volume of mineral oil and amplification was carried out using the "hot start" technique in a thermal cycler. The conditions for amplification involved denaturation at 94°C for 5 min followed by addition of Taq Polymerase. Thirty cycles were then performed: 94°C for 1 min, 64°C for 1 min, 72°C for 1 min and finally a 10 min extension at 72°C. The reaction mixture was then treated with exonuclease I to digest the primers not extended in the amplification step. This was achieved by incubating the PCR reaction mixture with 0.4 units of exonuclease I at 37°C for 30 min. The exonuclease was subsequently heat inactivated by incubating the reaction at 80°C for 15 min.

Uracil DNA-glycosylase (0.5 units) was then added and the incubation continued at 37°C for min.

Following treatment with uracil DNA-glycosylase, the AP sites generated in the amplified product were cleaved to completion by adding NaOH to a final concentration of 0.05M and heating the mixture for 15 min at 95°C. Under these conditions, cleavage occurs on the 5' side of each AP site. The reaction was then neutralised by addition to Tris base to 30mM final concentration. Both Exol and UDG are diluted containing 0.07M Hepes KOH pH 8.0, 1mM EDTA, 1mM DTT and 50% glycerol.

An equal volume of formamide loading dye (90% formamide, 0.025% Bromophenol blue, 0.025% Xylene cyanol) was added to the sample which was then heated at 85°C for 5 min. The sample was then loaded onto a 20% denaturing (7M urea) polyacrylamide gel and electrophoresis was carried out for 3-4 hours at 60W for size analysis of the cleaved products in the sample. Following electrophoresis, autoradiography was carried out by exposing the gel directly to X-ray photographic film for 12 hrs at -70°C. During the second phase of the genotyping, an improved protocol was used. Essentially, PCR amplification was carried out in 25 ml reactions, each of which contained 100 ng genomic DNA, PCR buffer (100 mM Tris-HCl pH 8.3 (20°C), 500 mM KCl, 15 mM MgCl₂), 200 mM of each dNTP, 300 nM of each primer and 0.5 U Taq polymerase (Boehringer). Conditions for amplification of exon 3 were 30 cycles at 94°C for 45 s, 61°C for 45 s, 72°C for 60 s using 5'-TACTCCCGAGTCTCCGGGTCTG-3' (SEQ ID NO. 1) as the forward primer and 5'-AGGCGCCCCACTGCCCCCTGGTAC-3' (SEQ ID NO. 2) as the reverse primer giving rise to the amplified C-93 allele (SEQ ID NO. 4) and amplified T-93 allele (SEQ ID NO. 5). All of the samples were then genotyped for the HLA-G C/T-93 polymorphism using the recently described glycosylase mediated polymorphism detection (GMPD) method (Vaughan & McCarthy, 1998).

Essentially a 319 bp fragment was amplified by semi-nested PCR from exon 3 using the forward primer 5'-GACCGAGGGGGTGGGGCCAGGTTCT-3' (SEQ ID NO. 3) and the reverse primer 5'-AGGCGCCCCACTGCCCTGGTAC-3' (SEQ ID NO. 1) giving rise to the amplified C-93 allele (SEQ ID NO. 6) and amplified T-93 allele (SEQ ID NO. 7). In the semi-nested amplification reaction dTTP was replaced by dUTP. The 3' end of the forward primer was designed so that the first U incorporated downstream of the forward primer was at, or distal to, the polymorphic site in codon 93. Following amplification using P³² end labelled forward primer, glycosylase mediated cleavage of the amplified product was performed. Cleavage products were resolved by denaturing gel electrophoresis (20% polyacrylamide) and visualised by autoradiography. The C-93 allele was detected as a 32 n fragment (SEQ ID NO. 8) and the T-93 allele as a 27 n fragment (SEQ ID NO. 9).

The common 14 base pair insertion / deletion polymorphism in exon 8 of the HLA-G gene is referred to as I/D-E8 herein (also known as where I-E8 is one allele of the polymorphism and D-E8 is the other allele of the polymorphism). Genotyping of the HLA-G exon 8 deletion polymorphism was performed by amplifying a short section flanking the deletion location in exon 8. This was achieved using the polymerase chain reaction with primers designed to hybridise to known DNA sequence in exon 8. The forward primer was 5'-TGTGAAACAGCTGCCCTGTGT-3' (SEQ ID NO. 10) and the reverse primer was 5'-AAGGAATGCAGTTCAGCATGA-3' (SEQ ID NO. 11).

The polymerase chain reaction was carried out in a total volume of 25µl, with 100ng genomic DNA, 50ng of each primer, 0.2mM of each deoxyribonucleoside triphosphate (dATP, dCTP, dGTP and dTTP) 50mM KCl, 10mM Tris-HCl, pH 9.0 at 25°C, 0.1% Triton X-100, 0.5mM MgCl and 0.5U of Taq Polymerase. Reaction mixtures were covered with an equal volume of mineral oil and amplification was carried out using the "hot start" technique in a thermal cycler. The conditions for amplification involved denaturation at 94°C for 5 min followed by addition of Taq Polymerase. Thirty cycles were then performed: 94°C for 1 min, 54°C for 1 min, 72°C for 1 min and finally a 10 min extension at 72°C. The I/D exon 8 polymorphism was genotyped by size separation of the PCR products on a 10% non denaturing polyacrylamide gel and visualised by staining with ethidium bromide, the I-E8 insertion allele giving rise to a 151 bp product (SEQ ID NO. 12) and the D-E8 deletion allele giving rise to a 137 bp product (SEQ ID NO. 13).

Allele specific genotyping. In order to gain more information from the transmission of HLA-G polymorphisms in the second phase of the work, allele specific genotyping was performed. In the majority of cases, maternal and paternal C/T-93 and I/D-E8 HLA-G haplotypes could be directly assigned. In cases where all members of a trio were heterozygous for either C/T-93 or I/D-E8 polymorphisms, allele specific amplification was performed in order to assign haplotypes. This was achieved using allele

specific primers which allowed selective amplification of the I-E8 or D-E8 allele. Following allele specific amplification, the C/T-93 polymorphism was then genotyped using the GMPD assay described above. Conditions for amplification of the I-E8 allele were 30 cycles at 94°C for 45s, 64°C for 45s, 72°C for 60s using 5'-TACTCCCGAGTCTCCGGGTCTG-3' (SEQ ID NO. 1) as the forward primer and 5'-CAAAGGGAAGGCATGAACAAATCTTG-3' (SEQ ID NO. 14) as the reverse primer. Conditions for amplification of the D-E8 allele were 30 cycles at 94°C for 45s, 56°C for 45 s, 72°C for 60s using 5'-TACTCCCGAGTCTCCGGGTCTG-3' (SEQ ID NO. 1) as the forward primer and 5'-GTTCTTGAAGTCACAAAGGGACTTG -3' (SEQ ID NO. 15) as the reverse primer. Such allele specific amplification gives rise to four possible haplotypes, namely I-E8 and C-93 haplotype (SEQ ID NO. 16), I-E8 and T-93 haplotype (SEQ ID NO. 17), D-E8 and C-93 haplotype (SEQ ID NO. 18) and D-E8 and T-93 haplotype (SEQ ID NO. 19).

All individuals in both sets of trios were genotyped for some or all of these polymorphisms (table 1 and 2). transmitted and non-transmitted alleles were assigned and haplotypes were constructed. All individuals were genotyped for the C/T-93 and I/D-E8 polymorphism. Comparative statistical analysis was performed. In the second phase of the work more elaborate analysis was performed. Comparison of allele and haplotype frequencies and genotype distribution for the polymorphisms between and within the sample cohorts, and was performed using chi-squared contingency table analysis and/or log linear model analysis and/or transmission disequilibrium testing.

In cases where all members of a trio were heterozygous for C/T-93 or I/D-E8 polymorphisms, allele specific amplification was performed to determine haplotypes and thus assign transmitted and non-transmitted alleles. This was achieved using primers which allowed specific amplification of a section of the HLA-G gene from either the insertion, or, deletion in exon 8 to a site 5' of codon 93. The C/T-93 polymorphism was then genotyped in the specifically amplified allele. Using this approach transmitted and nontransmitted alleles to offspring were assigned.

Results

All of the genetic studies to date apart from the one concerning the HLA-G deletion polymorphism have examined the genotype of the pre-eclamptic mother. We took the view that foetal HLA-G is the most likely candidate gene for pre-eclampsia. Pre-eclampsia trios where the offspring was the offspring of the primagravida pre-eclampsia pregnancy and a control group of offspring of normal primagravida pregnancies were studied. 54 pre-eclamptic offspring and 48 control offspring were included in the investigation

Genetic analysis of the HLA-G C/T-93 polymorphism in the pre-eclamptic offspring group revealed homozygosity for the C-93 allele in 7 cases (13%), homozygosity for the T-93 allele in 3 cases (5.6%) and heterozygosity in 44 cases (81.4%). In comparison, control offspring showed homozygosity for the C-93 allele in 18 cases (37.5%), homozygosity for the T-93 allele in 8 cases (16.6%) and heterozygosity in 22 cases (45.8%).

The frequencies of the C-93 and T-93 allele in the pre-eclamptic offspring were 0.537 and 0.463 respectively. In comparison, the frequencies in the control offspring were 0.604 and 0.396 respectively. The expected frequency distribution of the C-93 and T-93 alleles can be estimated with the formula $p^2 + 2pq + q^2 = 1$. With the allelic frequencies of $p=0.537$ and $q=0.463$ in the pre-eclamptic offspring group, the expected distribution of genotypes should be C-93/C-93 = 0.288, C-93/T-93 = 0.502, T-93/T-93 = 0.214. With the allelic frequencies of $p=0.604$ and $q=0.396$ in the control offspring group, the expected distribution of genotypes should be C-93/C-93 = 0.36, C-93/T-93 = 0.478, T-93/T-93 = 0.156. In comparison with the control group the distribution of genotypes in the pre-eclamptic offspring group is significantly different (Chi-square = 11.01, $p<0.001$, table 1).

It has been reported that no significant association was observed between the HLA-G exon 8 deletion polymorphism and pre-eclamptic offspring (Humphrey et al., 1995). In this work we also genotyped the pre-eclamptic and control offspring for the HLA-G deletion polymorphism. Genetic analysis of the HLA-G deletion polymorphism in the pre-eclamptic ($n=51$) and control offspring ($n=55$) groups revealed homozygosity for the normal allele in 12 cases (23.5%) and 13 cases (23.6%) respectively, homozygosity for the deletion allele in 8 cases (15.7%) and 12 cases (21.8%) respectively, and heterozygosity in 31 cases (60.8%) and 30 cases (54.5%) respectively.

The frequencies of the normal and deletion allele in the pre-eclamptic offspring were 0.539 and 0.46 respectively. In comparison, the frequencies in the control offspring were 0.509 and 0.491 respectively. In the pre-eclamptic offspring group, the expected distribution of genotypes should be normal allele/normal allele = 0.291, normal allele/deletion allele = 0.497, deletion allele/deletion allele = 0.212. The expected distribution of genotypes in the control offspring group should be normal allele/normal allele = 0.259, normal allele/deletion allele = 0.500, deletion allele/deletion allele = 0.241. In comparison with the control group the distribution of genotypes in the pre-eclamptic offspring group is not significantly different (Chi-square = 0.69, $p<0.30$, table 1).

The parents of the pre-eclamptic and control offspring were genotyped for the HLA-G C/T-93 genotype and the genotypes were analysed in conjunction with the offspring genotypes. In this analysis we scored the number of cases where the offspring had inherited a paternal C/T-93 allele that was not present in the maternal genotype. For the pre-eclamptic offspring, 41% of cases had a paternal allele of the C/T-93

genotype that was not present in the maternal genotype. By contrast, for the control offspring, 28% of cases had a paternal C/T-93 allele not represented in the maternal genotype.

Discussion

In this investigation, two common polymorphisms in the HLA-G gene in the offspring of pre-eclamptic and normal mothers were examined for association with pre-eclampsia. The two offspring groups are from Southern Ireland and are from the same ethnic background.

This is the first report determining association between pre-eclampsia in the mother and the foetal HLA-G genotype. Our results show a strong association between pre-eclampsia in the mother and heterozygosity for the C/T-93 polymorphism in offspring. This indicates that transmission of HLA-G alleles to offspring is different in normal offspring than in pre-eclampsia offspring. The result indicates that screening for susceptibility to pre-eclampsia can be achieved by genotyping of HLA-G in the mother and partner. Furthermore, since pre-eclampsia is associated with intrauterine growth retardation and miscarriage, it is likely that screening for susceptibility to intrauterine growth retardation, miscarriage and miscarriage-related infertility may also be achieved by HLA-G genotyping in the potential parents.

Following these results and to lend further support to this key finding, we expanded the number of subjects analysed to include additional normal primigravida trios where the mother had no history of pregnancy related problems, pre-eclampsia primigravida trios, families (mother, father, first and second offspring) where the mother had two or more successful normal pregnancies in the absence of pregnancy related disorders including pre-eclampsia and miscarriage, families (mother, father, first and second offspring) where the mother had pre-eclampsia in the first pregnancy and a normal second pregnancy in the absence of pregnancy related disorders including pre-eclampsia and miscarriage. As pre-eclampsia has been shown to be associated with miscarriage, we also included a cohort of recurrent miscarriage couples. The C/T-93 and I/D-E8 polymorphisms were genotyped in all individuals (Table 2 and Table 6), haplotypes were assigned and more elaborate statistics were applied to support our initial finding.

Linkage of HLA-G to pregnancy success in normal primigravidas

For transmission analysis of individual polymorphisms, we applied the log linear model and the transmission disequilibrium test. We also examined other polymorphisms in the HLA-G gene. The frequency of the A/T-31, A/T-107 and C/A-110 polymorphisms in the sample cohorts was 1.0/0.0, 0.95/0.05 and 0.94/0.06 respectively. For the analysis, we still utilised the commonly occurring C/T-93 and I/D-E8 polymorphisms. Using the allele specific amplification approach we assigned transmitted and nontransmitted alleles in the trios (Table 3). Since maternal genotype, foetal genotype (and specifically the paternal origin of foetal alleles) could potentially influence pregnancy outcome, a number of different

comparisons were made using this data. The log linear model of Weinberg et al. 1998 allows for causal scenarios in which the foetal genotype, parental genotypes or combinations thereof are directly relevant to risk. Maximum likelihood log-linear models of case-parent triad data were fitted. The log-linear model predicts the expected numbers of the 16 possible family types observed (for a bi-allelic marker), allowing for several "genetic risk factors" i.e. (a) whether the offspring carried one or more copies of an allele, (b) whether the mother carried one or more copies of an allele (c) a maternal origin effect and (d) a paternal origin effect (Table 4). The analysis was stratified on parental mating type assuming Hardy-Weinberg equilibrium. this corrects for the number of a particular allele that is found among the four parental alleles in a particular mating type, which has an obvious but uninteresting effect on allele distributions among the offspring. The advantage of this framework is that nested models of differing complexity are validly compared, starting with simple allelic effects and adding extra factors. We use a four-factor model, and then a stepwise reduced model which has removed the less significant factors automatically, thus providing a simpler model that accounts for important departures from expectations.

Fitting a four-parameter model to the data (Table 4) was significant for both the C-93 allele ($p=0.006$) and especially significant for the I-E8 allele ($p=0.0001$). Stepwise elimination of parameters which are not significant revealed that most of this can be accounted for by two effects: an effect due to foetal HLA-G alleles and, most strongly, by a parental origin effect for each allele (Table 4). Thus, both the C-93 and I-E8 alleles are significantly under-represented among offspring, and where they do occur, tend to be of paternal and maternal origin respectively. The maternal alleles in themselves are not significantly biased once these other effects are allowed for. The I-E8 allele is over 4 times more likely to be of maternal origin than expected (95% confidence interval 2.2-9.8).

When the transmission frequencies of maternal and paternal alleles to offspring were compared using chi-squared contingency table analysis, highly significant differences were observed for the C/T-93 and I/D-E8 alleles ($p_1=0.009$ and $p_1=0.000001$ respectively, Table 5, Table 3). This reflected a deficit in transmission of maternal C-93, D-E8 and paternal T-93, I-E8 alleles (Table 3). A significant difference was also observed between maternal and paternal non-transmitted I/D-E8 alleles showing that the maternal genotype plays a role in pregnancy outcome.

We verified that the highly significant findings from log-linear modelling by simpler comparisons. The transmission disequilibrium test transmission disequilibrium test assesses whether assess whether transmission of maternal and paternal alleles from heterozygous parents to offspring differed from the null expectation (of 50:50) and is valid even when Hardy-Weinberg equilibrium is violated by unusual population structure. When the transmission disequilibrium test was applied, the transmission of the C/T-93 and I/D-E8 alleles to offspring did not differ from the null expectation (Table 5). However, significant

deviations from the null expectation were observed when maternal and paternal transmission frequencies were analysed independently of each other.

Transmission of the maternal T-93 (chi-squared transmission disequilibrium test: $p=0.032$) and I-E8 (chi-squared transmission disequilibrium test: $p=0.0005$) alleles and also the paternal D-E8 allele (chi-squared transmission disequilibrium test: $p=0.01$) to offspring was markedly more frequent than expected (Table 5). There was an excess transmission of the maternal T-93 allele from heterozygous mothers to offspring. Specifically, thirty out of forty four offspring inherited the maternal T-93 allele from C/T-93 heterozygote mothers and forty out of fifty two offspring inherited the maternal I-E8 allele from I/D-E8 heterozygote mothers (Table 3). In addition, a contrasting excess transmission of the paternal D-E8 allele from heterozygote fathers to offspring (thirty one out of forty three cases) was observed (Table 3). These findings closely match the findings of the log-linear model.

We examined the data to determine if the transmission distortion could be accounted for by transmission to female or male offspring alone. However, there was no evidence that this was the case since of the forty out of fifty two offspring inheriting the maternal I-E8 allele from heterozygote mothers, 19 were females and 21 were males. Similarly, of the thirty one out of forty three offspring inheriting the paternal D-E8 allele from heterozygote fathers, 15 were females and 16 were males.

The primagravida mothers investigated here differed significantly from Hardy-Weinberg expectations ($p_1=0.006$) for I/D-E8 genotype frequencies (observed genotype frequencies: I/I; 17, I/D; 58, D/D; 15, expected I/I; 24, I/D; 45, D/D; 21). The transmission disequilibrium test results shows a significant effect without assuming Hardy-Weinberg equilibrium and thus support the log linear model results which was calculated assuming Hardy-Weinberg equilibrium.

Assignment of alleles transmitted and non transmitted to offspring was determined for five independent HLA-G polymorphisms permitting haplotype construction, and comparison of transmitted and non transmitted haplotypes. Thirteen haplotypes were observed (Table 6). Four of these, a-a-a-a-b, a-b-a-a-a, a-a-a-a-a and a-b-a-a-b, were relatively common. Differences between the frequencies of maternal and paternal transmission were apparent for all four common haplotypes indicating that the distortion of HLA-G allele transmission to primagravida offspring could still be accounted for by biases in transmission of the C/T-93 and I/D-E8 polymorphisms. We then constructed haplotypes for the C/T 93 and I/D-E8 polymorphisms alone for comparison purposes.

The C-D and T-I haplotype were most common (Table 7) The strength of linkage disequilibrium between the two markers is indicated by the high frequency of the C-D haplotype, for which the disequilibrium, expressed as a proportion of the maximum disequilibrium (D/D_{max}), is 0.344. Comparison of maternally

and paternally transmitted haplotypes to offspring using chi-squared contingency table analysis, revealed a highly significant difference between maternally and paternally transmitted haplotypes to offspring ($p_3=0.000003$) reflecting a deficit in transmission of both maternal haplotypes bearing the D-E8 allele (C-D and T-D) and both paternal haplotypes bearing the I-E8 allele (C-I and T-I) (Table 7). A significant difference was also observed between maternally and paternally non-transmitted haplotypes to offspring ($p_3=0.028$) showing that the maternal HLA-G genotype plays a role in pregnancy outcome.

Maternally and paternally transmitted haplotypes to individual offspring are shown in Table 8. The maternally transmitted T-I haplotype and the paternally transmitted C-D haplotype combination occurs in twenty one (34%) of the control offspring. By contrast the possible alternative combination (maternally transmitted C-D and paternally transmitted T-I) does not occur in any of the control offspring even though twenty two of the matings have this possibility.

In the primagravida offspring, homozygosity did not deviate from Hardy-Weinberg expectations. Comparison of the observed and expected number of homozygotes vs. heterozygotes within the offspring did not reveal any significant differences ($p_1 = 0.256$ for C/T-93 and $p_1 = 0.82$) showing that selection against homozygotes does not occur in primavidas.

The significant distortion observed for transmission of HLA-G alleles to primagravida offspring provides evidence for maternal and paternal allele specific HLA-G based selection of fetuses in normal primavidas. The selection observed was most pronounced for the HLA-G I/D-E8 polymorphism. The log linear model and transmission disequilibrium test analysis shows strong selection for the maternal I-E8 and paternal D-E8 allele in offspring. The selection effect is most dramatic for foetal combinations of the I/D-E8 alleles. In the total sample, thirty seven of the eighty four offspring have a maternal I-E8 paternal D-E8 allele combination. By contrast, there are only five offspring with the alternative maternal D-E8 paternal I-E8 allele combination (calculated from Table 8). These results show that maternal D-E8 paternal I-E8 fetuses are subject to significantly increased postzygotic prenatal loss and identify HLA-G as a key gene influencing this process. The deficiency of maternal D-E8 paternal I-E8 offspring is approximately 29% and closely matches postzygotic prenatal loss in prospective mothers which has been estimated to occur at a frequency of about 31% and in more than 20% of these cases, such loss occurs very early in pregnancy and is clinically unrecognisable.

The dichotomous effect whereby the maternal HLA-G I-E8 allele seemingly imparts a protective effect to the foetus while the equivalent paternal allele is detrimental is somewhat suggestive of genomic imprinting, however reports to date indicates that imprinting does not occur at the HLA-G locus.

It is not clear why there is an excess of heterozygote mother for the I/D-E8 genotype. The excess of heterozygote mother for the I/D-E8 genotype the population of mothers investigated have been selected on the basis of normal pregnancy outcome and as such represent a select group of the female population since the whole female population would include several other categories of females including infertile women (10-15% incidence), women that had a miscarriage (10-15% incidence) or pre-eclampsia (5-10% incidence).

In the primagravida offspring, homozygosity did not deviate from Hardy-Weinberg expectations. Comparison of the observed and expected number of homozygotes vs. heterozygotes within the offspring did not reveal any significant differences ($p_1 = 0.45$ for C/T-93 and $p_1 = 0.81$ I/D-E8) indicating that selection against homozygotes does not occur in primagravidas. The preferential transmission of a maternal I-E8 and paternal D-E8 HLA-G alleles to offspring might be expected to result in increased heterozygosity in the offspring. However, as the selection of offspring appears to be for maternal I-E8 and paternal D-E8 HLA-G allele combinations and against paternal I-E8 and maternal D-E8 allele combinations, the excess of the former heterozygote will be balanced by the deficiency of the latter.

As linkage disequilibrium occurs across the HLA locus and the results are proof that HLA-G and/or a HLA-G linked gene, cause the selection effects observed here. The C/T-93 polymorphism is a silent mutation while the I/D-E8 polymorphism occurs in the 3' untranslated region (UTR) of the gene. These polymorphisms have been considered innocuous. However, the evidence indicates that the deletion polymorphism has a functional effect on the HLA-G gene. The 14bp sequence of I/D-E8 polymorphism is largely conserved in primates and in the 3' UTR and/or in the last intron of HLA-B, C, J, A and E. 11 of the 14bp of the polymorphism is repeated in intron seven of the HLA-G gene. The core sequence "atttgt" is repeated one or more times in the 3' UTR of all class I genes but is absent in coding sequences.

Examination of the secondary structure around the I/D-E8 polymorphism using the mfold programme (Zuker, 1994) shows that the 14n sequence is involved in a region of the 3'UTR having extensive secondary structure and that the secondary structure is altered depending on the presence or absence of the 14n sequence (data not shown). Thus the presence or absence of the polymorphism may affect the stability and/or alternative splicing of HLA-G mRNA through formation of alternative secondary structures.

Examination of human preimplantation blastocysts showed that only 40% of the blastocysts expressed HLA-G and such expression was associated with an increased cleavage rate by comparison with embryos lacking the HLA-G transcript. Thus, polymorphisms affecting expression of HLA-G are likely to influence the rate of postzygotic prenatal loss by altering the cleavage rate in the embryo.

Taken together, the results provide evidence that different HLA-G alleles and/or combinations thereof and/or variations in DNA in linkage disequilibrium with HLA-G in the foetus and/or one or both parents of the foetus are responsible for postzygotic prenatal loss which may manifest as miscarriage or undetectable early miscarriage which would manifest as unexplained infertility.

5 Identification of HLA-G as the Pre-eclampsia gene

A cohort of pre-eclampsia primagravida trios (mother, father and first offspring) were identified in maternity hospitals, sampled and genotyped for the following polymorphisms in the HLA-G gene: C/T at codon 93 (C/T-93) (Table 9), A/T at codon 107 (A/T 107), C/A at codon 110 (C/A 110), and the insertion / deletion polymorphism in the non-translated region of the gene in exon 8 (I/D-E8) (Table 9).

10 Alleles transmitted and non transmitted to offspring were assigned (Table 10 and Table 11).

HLA-G genotypes and haplotypes in pre-eclampsia trios were examined independently using transmission segregation analysis. pre-eclampsia trios were also compared to the cohort of control primagravida trios. A significant difference in C/T-93 allele frequency was observed between control and pre-eclampsia mothers ($p_1 = 0.03$, Table 12). A significant difference was also observed for the allele frequency of the I/D-E8 polymorphism between control and pre-eclampsia fathers ($p_1 = 0.02$, Table 12). The frequency of the 93-E8 haplotypes differed significantly between control and pre-eclampsia mothers ($p_3 = 0.03$), control and pre-eclampsia fathers ($p_3 = 0.008$, Table 12) and also between control and pre-eclampsia offspring ($p_3 = 0.03$, Table 12).

The distribution of C/T-93 genotypes differed markedly between control and pre-eclampsia trios (Table 12), with a highly significant difference being observed between control and pre-eclampsia offspring ($p_2 = 0.001$), between control and pre-eclampsia fathers ($p_2 = 0.02$), and also between control and pre-eclampsia mothers ($p_2 = 0.05$). These differences reflected a significant excess of C/T-93 heterozygotes over Hardy-Weinberg equilibrium expectations in both pre-eclampsia offspring ($p_1 = 0.0002$) and pre-eclampsia fathers ($p_1 = 0.021$). A significant excess of I/D-E8 heterozygotes over Hardy-Weinberg expectations was also observed in control mothers, pre-eclampsia offspring and in pre-eclampsia fathers (Table 12).

Analysis of HLA-G haplotype sharing between offspring and mothers was also performed. No significant difference between pre-eclamptic cases and controls was observed for foetal-maternal sharing of HLA-G alleles or for sharing of the paternally transmitted HLA-G allele. There was no significant difference observed for offspring sex between controls and pre-eclampsia cases

Comparison of the frequency of maternally and paternally transmitted alleles and haplotypes to control and pre-eclampsia offspring revealed significant differences (Table 12). In particular, these differences showed an excess of the maternally inherited T-93/I-E8 haplotype, and paternally inherited C-93/D-E8 haplotype and a deficiency of the maternally inherited C-93/D-E8 haplotype, and paternally inherited T-93/I-E8 haplotype in control offspring by comparison with pre-eclampsia offspring (Table 10). Furthermore, there was a significant difference between non-transmitted maternal I/D-E8 alleles and 93-E8 haplotypes showing that the maternal non-transmitted alleles are associated with pregnancy outcome.

Only twelve out of fifty two control offspring inherited the maternal D-E8 allele from heterozygous (I/D-E8) mothers (Table 3). By contrast, the maternal D-E8 allele was transmitted to twenty one of thirty six pre-eclampsia offspring (Table 11). Taken together, these findings show a significant deficit of maternal D-E8 transmission to control offspring and a contrasting excess of maternal D-E8 transmission to pre-eclampsia offspring.

Further analysis within control and pre-eclampsia trios was achieved by comparison of maternally and paternally transmitted alleles and haplotypes to the offspring. In control offspring a highly significant difference was observed between transmission of maternal and paternal C/T-93 alleles (Table 5, $p_1=0.009$, calculated from Table 3). This reflected a deficit of maternal C-93 and paternal T-93 transmitted alleles (Table 3). A highly significant difference was also observed between transmission of maternal and paternal I/D-E8 alleles (Table 4, $p_1=0.000001$, calculated from Table 3), showing a deficit of transmission of maternal D-E8 and paternal I-E8 alleles to the offspring (Table 3). A significant, but contrasting difference between transmission of maternal and paternal alleles was present in pre-eclampsia offspring where an excess of transmission of maternal D-E8 and paternal I-E8 alleles, maternal C-93 and paternal T-93 was observed (Table 13, Table 10). A significant difference was present between maternally and paternally transmitted haplotypes in both control and pre-eclampsia offspring ($p_3=0.000003$, Table 5 and $p_3=0.005$ respectively) showing a deficit in transmission of maternal C-93/D-E8 haplotypes and paternal T-93/I-E8 haplotypes to the control offspring and an excess in transmission of maternal C-93/D-E8 haplotypes and paternal T-93/I-E8 haplotypes to the pre-eclampsia offspring (Table 10).

Maternally and paternally transmitted haplotypes to individual offspring are shown in Table 14. The maternally transmitted C-93/D-E8 haplotype and the paternally transmitted T-93/I-E8 haplotype combination occurs in twenty two (35%) of the pre-eclampsia offspring but does not occur in any of the control offspring. By contrast, the paternal C-93/D-E8, maternal T-93/I-E8 haplotype combination occurs in both control and pre-eclampsia offspring but is in excess in the control offspring. This finding

provides evidence that combinations of HLA-G alleles / haplotypes in the foetus are causative of pre-eclampsia in the mother

Taken together, the results provide evidence that different foetal HLA-G alleles and/or combinations thereof and/or variations in DNA in linkage disequilibrium with HLA-G in the foetus and/or one or both parents of the foetus are responsible for pre-eclampsia

Association of HLA-G with Recurrent Miscarriage

A cohort of couples where the mother had three or more consecutive miscarriages were identified, sampled and genotyped for the C/T-93 and I/D-E8 polymorphisms in the HLA-G gene and 93-I-E8 haplotypes were assigned. RSA mothers and RSA fathers were compared to the cohort of control and pre-eclampsia primagravida trios for C/T-93 and I/D-E8 allele frequency, C/T-93 and I/D-E8 genotype distribution and 93-E8 haplotype frequency. The genotypes and haplotypes of the couples are shown in Table 16.

A significant difference in C/T-93 allele frequency was observed between control and pre-eclampsia mothers ($p_1 = 0.03$) and control and RSA mothers ($p_1 = 0.002$) but not between pre-eclampsia and RSA mothers. The frequency of the 93-E8 haplotypes differed significantly between control and pre-eclampsia mothers ($p_3 = 0.03$), control and RSA mothers ($p_3 = 0.01$), but not between pre-eclampsia and RSA mothers.

The 93-E8 haplotypes of female and male mating partners were constructed. 50% of couples have the possibility of producing foetuses with the maternally transmitted C-93/D-E8 haplotype and the paternally transmitted T-93/I-E8 haplotype combination. This compares with a 24% possibility in control couples and a 44% possibility in pre-eclampsia couples. The possibility of producing foetuses with the maternally transmitted D-E8 allele and the paternally transmitted I-E8 allele is 46% for control couples, 70% for pre-eclampsia couples and 85% for recurrent miscarriage couples. Maternally transmitted C-93/D-E8 haplotype and the paternally transmitted T-93/I-E8 haplotype combinations in foetuses are only found in pre-eclampsia offspring. Recurrent miscarriage couple no. 15 must produce such a foetus. Taken together, the results provide evidence that foetal genotype associated with pre-eclampsia are also associated with miscarriage. Finally, mating couples where the female is homozygous for the T-93/I-E8 haplotype and the male has the C-93/D-E8 and T-93/I-E8 haplotypes were found in seven of sixty three pre-eclampsia cases and were absent in controls. Two of twenty of the recurrent miscarriage mating couples (no. 10 and 13, Table 17) also had the same haplotype combinations. This provides evidence that partners where the female is homozygous for the T-93/I-E8 haplotype and the male has the C-93/D-E8 and T-93/I-E8 haplotypes are susceptible to pre-eclampsia and/or miscarriage. Taken together, the results

provide evidence that miscarriage and pre-eclampsia are closely related and that miscarriage is a severe expression of PE. One offspring of T-93/I-E8 and C-93/D-E8 haplotype combination in control trios was found where the mother was homozygous for T-93/I-E8 and father had the C-93/D-E8 haplotype and the C-93/I-E8 haplotype. One offspring of T-93/I-E8 and T-93/I-E8 haplotype combination in control trios was also found where the mother was homozygous for T-93/I-E8 and father had the T-93/I-E8 haplotype and the C-93/I-E8 haplotype. This result shows that the non-transmitted male haplotype has a major influence on pregnancy outcome and indicates that sperm/semen contains a factor which influences susceptibility to pre-eclampsia and miscarriage.

Taken together, the results provide evidence that different HLA-G alleles and/or combinations thereof and/or variations in DNA in linkage disequilibrium with HLA-G in the foetus and/or one or both parents of the foetus are responsible for miscarriage.

Induction of tolerance to HLA-G pre-eclampsia/miscarriage haplotypes in first pregnancy

The results show that maternally transmitted D-E8 allele and the paternally transmitted I-E8 to offspring are linked to pregnancy outcome and that the maternally transmitted C-93/D-E8 haplotype and the paternally transmitted T-93/I-E8 haplotype combination cause pre-eclampsia and miscarriage in primagravidas. We analysed HLA-G transmission in fifty three couples that have had two successful pregnancies without a history of miscarriage or PE.

The possibility of producing foetuses with the maternally transmitted D-E8 allele and the paternally transmitted I-E8 allele was 15% for this cohort of normal couples. The results thus show a clear correlation between successful pregnancy outcome and the probability of possibilities of producing foetuses with the maternally transmitted D-E8 and the paternally transmitted I-E8 HLA-G alleles. Recurrent miscarriage - 85%, pre-eclampsia - 70%, first pregnancy normal - 46%, first and second pregnancy normal 15%.

The maternally transmitted C-93/D-E8 haplotype and the paternally transmitted T-93/I-E8 haplotype combination cause pre-eclampsia and miscarriage in primagravidas. We determined if primagravida normal pregnancy induced tolerance to the foetal pre-eclampsia / miscarriage haplotype combinations when they occur in pregnancy two. There were no first offspring detected bearing the maternally transmitted C-93/D-E8 haplotype and the paternally transmitted T-93/I-E8 haplotype combination. There were five second offspring bearing the maternally transmitted C-93/D-E8 haplotype and the paternally transmitted T-93/I-E8 haplotype combination (Table 18). We also determined if primagravida pre-eclampsia pregnancy induced tolerance to the foetal pre-eclampsia / miscarriage haplotype combinations when they occur in pregnancy two. We analysed nine families where the mother suffered pre-eclampsia in

her first pregnancy and had a normal second pregnancy. There were three second offspring bearing the maternally transmitted C-93/D-E8 haplotype and the paternally transmitted T-93/I-E8 haplotype combination in the absence of pre-eclampsia even though the same combination caused pre-eclampsia in the first pregnancy (Table 19).

5 This proves that tolerance to the paternal antigens in the foetus is induced in the first pregnancy. More specifically, tolerance to the problematic maternally transmitted C-93/D-E8 haplotype and the paternally transmitted T-93/I-E8 haplotype was induced in first pregnancy. Thus exposure to HLA-G alleles and/ or combinations thereof and/or paternal antigens presented to the maternal immune system by HLA-G in the first pregnancy induces tolerance to the pre-eclampsia / miscarriage haplotype combination so that these
10 problematic haplotypes can occur in the second pregnancy without associated pre-eclampsia or miscarriage.

The genetic linkage, association and correlation approaches used in the large number of subject cohorts provides proof that HLA-G is a susceptibility gene for normal pregnancy, pre-eclampsia and miscarriage. As pre-eclampsia is associated with intra uterine growth retardation, the HLA-G gene is also a
15 susceptibility gene for intra uterine growth retardation. As miscarriage frequently occurs so early that it is not detected, the HLA-G is also a susceptibility gene for miscarriage related unexplained infertility. Exposure to foetal antigens including HLA-G in the first pregnancy has been shown to induce tolerance to antigens that are problematic in first pregnancy and thus provide a means for potential treatment of pre-eclampsia, miscarriage, intrauterine growth retardation, miscarriage related infertility and autoimmune
20 disease and provide a means to induce tolerance to foreign antigens for purposes such as transplantation of foreign tissue.

The HLA-G I/D-E8 polymorphism has been investigated previously in pre-eclampsia and no detectable relationship was observed between susceptibility to pre-eclampsia and HLA-G (24). This result is consistent with the results reported here in that an HLA-G effect is not seen when the I/D-E8
25 polymorphism is independently analysed by association studies alone.

The results presented here show that genetic screening of maternal and/or paternal and/or foetal DNA is of value for predictive testing of susceptibility to pre-eclampsia, eclampsia, intrauterine growth retardation, miscarriage and miscarriage-related infertility. Furthermore, transmission of HLA-G alleles to offspring in normal pregnancy differs from the normal expectation. Therefore, the results presented
30 here show that genetic screening of maternal and/or paternal foetal DNA is of value for predictive testing of susceptibility to normal pregnancy

Preferably, foetal nucleic acid is isolated from any material containing nucleic acid of foetal origin in the mother such as amniotic fluid, maternal blood or chorionic villus. Furthermore, the results show that genetic screening of parents will also be of value for predictive testing of susceptibility to pre-eclampsia.

Although the function of HLA-G apart from its role in regulating NK cell activity and induction of IL-3 and IL-1 beta in PBMCs is as yet poorly understood, HLA-G is an excellent candidate for pre-eclampsia since HLA-G is considered to play a key role in foetal-maternal immune interactions. The C/T-93 HLA-G allele associated with pre-eclampsia is a silent polymorphism but its effect on HLA-G mRNA stability or splicing is unknown. It is likely that this polymorphism and/or variations linked to this polymorphism play a causative role in pre-eclampsia.

In this work, we have demonstrated a difference between pre-eclamptic and control offspring with respect to sharing of the paternal C/T-93 allele between the offspring and their mothers. This result indicates that pre-eclampsia may arise due to the presence of a HLA-G haplotype in the foetus that has not previously been encountered by the mother. Since HLA-G is in tight linkage disequilibrium with the HLA locus, it is likely that the paternal HLA-G itself and/or the presence of an extended paternal HLA haplotype in the foetus that has not previously been encountered by the mother causes pre-eclampsia. Furthermore, since HLA-G is in tight linkage disequilibrium with the HLA locus, determination of the extended paternal HLA haplotype segregating in the foetus and comparison of the haplotype with the maternal HLA haplotypes will allow diagnosis of susceptibility to pre-eclampsia.

While other associations have been reported between pre-eclampsia and the maternal genotype, the results reported here are much more consistent with epidemiological studies on pre-eclampsia. In particular, the association between a foetal HLA-G genotype is consistent with the observation that a) pre-eclampsia is more common in sisters than in the normal population, b) pre-eclampsia is discordant in identical twin mothers and c) pre-eclampsia can occur with a change of male partner. Pre-eclampsia is rare in second or later pregnancies indicating that initial exposure to functional HLA-G prevents pre-eclampsia. In addition, HLA-G is now known to induce synthesis IL-3 and IL-1 beta and down-regulate tumour necrosis factor-alpha production. These observations coupled with the results presented here indicates that HLA-G protein, IL-3 and/or IL-1 beta or inhibitors of tumour necrosis factor-alpha will be useful for treatment of intrauterine growth retardation, pre-eclampsia, miscarriage and miscarriage-related infertility.

The HLA-G genotype associated with pre-eclampsia and miscarriage is likely to have one of a small number of consequences:

- i) it could result in reduced expression of HLA-G which would be reflected as decreased levels of cellular and/or soluble HLA-G (the HLA-G primary transcript is alternatively spliced to yield several

different mRNAs. One of these alternatively spliced forms includes intron 4. The open reading frame in this mRNA continues into intron 4, terminating 21 amino acids after the alpha3 domain - encoded by exon 4. Thus, the transmembrane region encoded by exon 5 and the cytoplasmic tail of HLA-G is excluded. The resultant protein is hence soluble). Thus measuring of cellular and/or soluble HLA-G levels and comparing these levels with the normal observed levels would allow one to diagnose susceptibility to pre-eclampsia and miscarriage;

ii) the HLA-G genotypes associated with pre-eclampsia may lead to variations in HLA-G mRNA and/or HLA-G protein which in turn could be detected by characterisation of HLA-G mRNA and/or protein.

Thus, characterisation of HLA-G protein in pregnant females, fetuses and/or respective male mating partner would allow one to diagnose susceptibility to pre-eclampsia and miscarriage;

iii) expression of the HLA-G protein leads directly or indirectly to alterations in the levels of certain molecules such as IL-3, IL-1 beta and/or tumour necrosis factor alpha. The HLA-G genotypes associated with pre-eclampsia may result in changed expression of such molecules. Thus measuring of the levels of such molecules and comparing these levels with the normal observed levels would allow one to diagnose susceptibility to pre-eclampsia and miscarriage;

iv) The HLA-G genotypes associated with pre-eclampsia may result in decreased expression of HLA-G. This in turn would lead to increased lysis of trophoblasts by NK cells. Thus measuring of the levels of trophoblast specific marker and comparing these levels with the normal observed levels would allow one to diagnose susceptibility to pre-eclampsia and miscarriage.

The HLA-G variants associated with pre-eclampsia and miscarriage and normal pregnancy are likely to have one of a small number of consequences:

i) a variant could result in altered expression of HLA-G splice forms and levels thereof which would be reflected as altered levels of HLA-G splice forms including soluble HLA-G in the serum.. Thus measuring of size, levels and/or splice forms of HLA-G mRNA and/or protein including soluble HLA-G levels and comparing these levels with the normal observed levels would allow one to diagnose susceptibility to pre-eclampsia and miscarriage;

ii) the HLA-G variants associated with pre-eclampsia and miscarriage may result in variations in HLA-G protein which in turn could be detected by protein characterisation of cellular and/or soluble HLA-G.

Thus characterisation of HLA-G protein in pregnant females, fetuses and/or respective male mating partner would allow one to diagnose susceptibility to pre-eclampsia and miscarriage;

iii) expression of the HLA-G protein leads directly or indirectly to alterations in the levels of certain molecules such as IL-3, IL-1 beta and/or tumour necrosis factor alpha. The HLA-G variants associated with pre-eclampsia may result in changed expression of such molecules. Thus measuring of the levels of such molecules and comparing these levels with the normal observed levels would allow one to diagnose susceptibility to pre-eclampsia and miscarriage;

iv) the HLA-G variants associated with pre-eclampsia and miscarriage may result in increased or decreased expression of paternal and/or maternal HLA-G. This in turn would lead to increased lysis of trophoblasts by NK cells and/or cytotoxic T cells. Thus measuring of the levels of trophoblast specific marker and comparing these levels with the normal observed levels would allow one to diagnose

5 susceptibility to pre-eclampsia.

v) the HLA-G variants associated with pre-eclampsia and miscarriage may result in increased or decreased cell cleavage rates in the embryo. Thus measuring of cell cleavage rates in cells expressing one or more HLA-G variants and any combinations thereof would allow one to diagnose susceptibility to pre-eclampsia and miscarriage.

10 The results show that HLA-G polymorphism plays a major role in predisposition to normal, pre-eclampsia and miscarriage outcome in pregnancy and that haplotypic combinations and parent-of-origin effects mediate the influence of HLA-G polymorphism on these outcomes. The results show a strong association between foetal and paternal HLA-G genotypes and PE, and analysis of heterozygote v. homozygote mating outcomes indicate that transmission of HLA-G alleles to the pre-eclampsia offspring, but not to control offspring, is distorted. The results provide evidence for linkage of the maternal HLA-G I-E8 allele to normal pregnancy outcome in primagravidas and the observed deficit of maternal D-E8 allele and C-93/D-E8 haplotype transmission to control offspring indicates selection for foetuses on the basis of HLA-G genotype in primagravida normal pregnancies. The transmission distortion of the maternal D-E8 allele to the foetus indicates that the effect seen in normal primagravidas is mediated by the maternal allele acting primarily in the foetus. Thus, the maternal HLA-G imparts a protective effect to the foetus which enhances normal pregnancy outcome. This finding indicates that maternal selection of the HLA-G I-E8 and other protective HLA-G alleles occurs in normal pregnancy. By contrast, the maternal D-E8 allele was prevalent in heterozygous pre-eclampsia offspring, indicating that susceptibility to pre-eclampsia partly arises through the lack of a protective maternal HLA-G allele in the foetus. The chi-squared contingency table analysis agreed with the log linear model analysis in that the C-93 allele was over-represented in pre-eclampsia offspring and a bias towards maternal inheritance of I-E8 was present in controls. Furthermore, the log linear model showed that the foetal C-93 allele is under-represented in control offspring with a strong bias towards paternal inheritance of the allele. This indicates that the paternal C-93 allele also imparts a protective or alternatively does not introduce a problematic effect to the foetus which improves the prospect of a normal pregnancy outcome. These results are in good agreement with the findings observed when maternal and paternal haplotype combinations were constructed for individual control and pre-eclampsia offspring where more than one third of the pre-eclampsia cases had a maternal C-93/D-E8 paternal T-93/I-E8 haplotype combination that was absent in the controls. Taken together, the data indicate a strong association between both maternal and paternal HLA-G alleles acting through the foetus and normal pregnancy outcome and indicate that pre-eclampsia

arises through the absence of protective maternal and protective or problematic paternal HLA-G alleles in the foetus. Furthermore, considering that there are likely to be several HLA-G alleles with functional differences, and as more than one third of pre-eclampsia cases can be accounted for by a particular maternal / paternal haplotype combination, the results show that the magnitude of the effect of HLA-G in normal and pre-eclampsia pregnancies is large.

Alternatively, a protective foetal-maternal HLA-G allele is likely to arise through the transmission of a dominant maternal allele to the foetus which is recognised as self by the maternal immune system. A protective foetal-paternal allele is likely to arise through cross recognition of the paternal allele as self by the maternal immune system. A problematic foetal-paternal allele is likely to arise through cross recognition of the paternal allele as non- self by the maternal immune system. The results indicate maternal education of the lymphocyte repertoire for maternal HLA-G during and/or prior to pregnancy and for paternal HLA-G during pregnancy. The results also indicate certain paternal HLA-G alleles are compatible with the maternal immune system while others are less compatible. Combinations of less compatible/incompatible paternal HLA-G alleles with maternal alleles which do not protect against the paternal alleles are likely to cause susceptibility to pre-eclampsia and miscarriage.

The fact that second offspring of primagravida normal and pre-eclampsia mothers have the maternal C-93/D-E8 paternal T-93/I-E8 genotype in the absence of pre-eclampsia in the second pregnancy is evidence that maternal education for foetal-paternal antigens occurs during the first pregnancy and that this education is mediated by HLA-G. It is clear from this work that the polymorphisms analysed and/or closely linked polymorphisms in HLA-G or flanking HLA genes contribute directly to enhancing normal pregnancy outcome and to susceptibility to pre-eclampsia and miscarriage. One likely explanation may be that the polymorphisms reported here destabilise HLA-G mRNA and/or alter the splicing pattern and/or glycosylation pattern of HLA-G. The presence or absence of polymorphism is likely to effect the stability and/or alternative splicing of HLA-G mRNA. Thus a protective foetal-maternal HLA-G allele is likely to arise through the transmission of a maternal allele to the foetus which may or may not be expressed in the embryo. A protective foetal-paternal allele is likely to arise through the transmission of a paternal allele to the foetus which may or may not be expressed in the embryo.

At least twelve different haplotypes have been described for the HLA-G gene. Considering the link observed between HLA-G and recurrent miscarriage, it is likely that the combination of HLA-G alleles in the early foetus and/or the combination of the HLA-G alleles in the mother has serious effects on the outcome of implantation in general and is likely to account for cases of unexplained or idiopathic infertility as well as miscarriage. The previously reported link between pre-eclampsia and intra-uterine

growth retardation indicates that the latter is also likely to be linked to parent of origins effects of foetal HLA-G alleles and indicate that maternal HLA-G alleles also play a role in the foetal growth outcome.

HLA-G is capable of protecting otherwise susceptible target cells from natural killer cell mediated lysis through its interaction with inhibitory receptors on natural killer cells. HLA-G is also capable of stimulating an HLA-G restricted lymphocyte response. HLA-G molecules can serve as target molecules in lytic reactions with lymphocytes, and HLA-G is involved in education of the lymphocytic repertoire. Thus, pre-eclampsia, miscarriage, miscarriage-related infertility and intrauterine growth retardation is likely to arise through a mechanism involving blood mononuclear cells such as natural killer cells and cytotoxic T lymphocytes whereby interaction between the female mating partner's T cells and foetal antigens is compromised by comparison with normal pregnancy. Thus, compromised interaction leading to the lack of tolerance leads to cell killing. Compromised interaction also can lead to lack of stimulation of cells expressing HLA-G molecules and/or lack of stimulation of cells interacting with cells expressing HLA-G molecules. The fact that the maternal C-93/D-E8 paternal T-93/I-E8 HLA-G genotype can occur in the second pregnancy of a primagravida pre-eclampsia case without pre-eclampsia indicates that education mediated by foetal HLA-G to foetal antigens occurs in the first pregnancy of such mothers which overcomes compromised interactions in second and subsequent pregnancies. The fact that a deficit of maternal C-93/D-E8 genotypes and an excess of T-93/I-E8 genotypes are transmitted to control offspring but not to pre-eclampsia offspring indicates that selection for foetuses that express antigens for which the mother is educated occurs in normal pregnancy. The fact that pre-eclampsia rarely occurs in a second pregnancy when the first pregnancy has been normal indicates that induction of education to foetal antigens mediated by HLA-G also occurs during and prior to the first pregnancy in normal mothers and that pre-eclampsia, miscarriage, miscarriage-related infertility and intra-uterine growth retardation arises from lack of education and/or inadequate induction of education to the foetal antigens in the female mating partner during and/or prior to pregnancy. Lack of and/or compromised induction of education to paternal antigens such as HLA-G in the foetus and/or a defective HLA-G interaction with natural killer cells could lead to lysis of trophoblasts and/or lack of stimulation of trophoblasts leading to reduced trophoblast function and/or lack of stimulation of cells interacting with trophoblasts. Thus, HLA-G linked conditions such as pre-eclampsia, miscarriage, miscarriage-related infertility and intrauterine growth retardation are likely to arise through blood mononuclear cell mediated killing of accessible foetal tissues such as trophoblasts and/or lack of stimulation of trophoblastic cells because of compromised HLA-G interaction with blood mononuclear cells trophoblasts and/or lack of stimulation of blood mononuclear cells because of compromised HLA-G interaction with trophoblastic cells. Since major histocompatibility (MHC) molecules like HLA-G interact with blood mononuclear cells including cytotoxic T cells and natural killer cells, there is likely to be abnormal interaction between maternal blood mononuclear cells and foetal cells presenting MHC / MHC-antigen complexes and/or MHC / MHC-antigen complexes

secreted from foetal cells in pre-eclampsia, miscarriage and intra-uterine growth retardation by comparison with normal pregnancies. Thus, the blood mononuclear cell response and/or the trophoblast response to such an interaction is likely to be abnormal in the HLA-G associated disorders. In particular, the cytokine response produced as a result of such an interaction is likely to be abnormal by comparison with the normal situation.

Thus, diagnosis of susceptibility to pre-eclampsia, miscarriage, miscarriage-related infertility and intrauterine growth retardation and prediction of pregnancy outcomes may be achieved by direct and indirect measurement of the education in the female mating partner to foetal antigens and/or direct and indirect measurement of the interaction between blood mononuclear cells and HLA-G and/or HLA-G expressing cells. Furthermore, direct and indirect measurement of the education in the female mating partner to foetal antigens and/or direct and indirect measurement of the natural killer cell activity in the female mating partner to HLA-G expressing cells and/or direct and indirect measurement of the interaction between blood mononuclear cells and HLA-G and/or HLA-G expressing cells offers a means to monitor the course of pregnancy.

Induction of education to foetal antigens in the female mating partner by treatment with HLA-G and/or peptides known to bind to HLA-G constitutes a therapeutic means for prevention and/or treatment of pre-eclampsia, miscarriage, miscarriage-related infertility and intrauterine growth retardation and any other HLA-G related disorders.

The finding that combinations of HLA-G variants in the foetus are closely associated with pre-eclampsia and miscarriage coupled to the fact that HLA-G interacts with blood mononuclear cells offers a further means to prevent and/or treat pre-eclampsia miscarriage, miscarriage-related infertility and intrauterine growth retardation by inhibition and/or alteration of the interaction of HLA-G and/or HLA-G variants with blood mononuclear cells. This may be achieved by any one or combination of approaches including treatment with one or more molecules which recognise HLA-G and/or variants of HLA-G and/or one or more HLA-G receptors on blood mononuclear cells, and/or inactivation of the HLA-G gene and/or HLA-G gene variants and/or one or more HLA-G receptors on blood mononuclear cells. For example, this would be achieved by treatment with HLA-G specific and/or HLA-G receptor specific antibodies which interfere with HLA-G - blood mononuclear cell interaction and/or treatment with one or more enzymes which recognise and alter HLA-G and/or HLA-G receptors on blood mononuclear cells and/or treatment with one or more peptides which bind to HLA-G and/or HLA-G receptors on blood mononuclear cells. Alternatively, inhibition of the interaction of one or more HLA-G variants with blood mononuclear cells may be achieved by inactivating the HLA-G gene or HLA-G gene variant and/or one or more HLA-G receptors on blood mononuclear cells. This may be achieved through the use of one or more gene

inactivating approaches such as treatment with one or more nucleic acid antisense and/or ribozyme molecules which inhibit expression of the HLA-G gene and/or HLA-G gene variant and/or one or more HLA-G receptors on blood mononuclear cells. This may be also be achieved by inactivating the HLA-G gene in one or both partners of a mating couple somatically or in the germ line through the use of gene therapy approaches whereby inhibitory nucleic acid based molecules such as antisense, and/or ribozyme are introduced into an individual. This may be also be achieved by inactivating the HLA-G gene in one or both partners of a mating couple somatically or in the germ line through the introduction of all or part of a HLA-G gene in such a way that it recombines with the endogenous HLA-G in the cell and inactivates it. Alternatively, the HLA-G gene and/or variants of the HLA-G gene and/or any of it's receptors may be employed in gene therapy methods in order to increase the amount of expression products of such genes in an individual allowing compensation of any deficiency of HLA-G and/or it's receptors in an individual. Thus, alteration of the interaction of HLA-G and/or HLA-G variants with blood mononuclear cells may be achieved by introduction of one or more HLA-G gene variants into somatic cells and/or into the germline of one of both partners of a mating couple or into the fertilised egg or cells arising from the fertilised egg prior to implantation. This is of particular importance for increased fertility for animal breeding purposes. For example, introduction of one or more HLA-G gene variants into the germline of one of both partner of a mating couple or into the fertilised egg or cells arising from the fertilised egg where the HLA-G variant is compatible with the prospective mother offers a means to improve fertility and pregnancy outcome arising from any incompatibility between foetal HLA-G and maternal cells in the mother.

HLA-G binds a diverse but limited array of peptides in a manner similar to that found for classical class I molecules and it has been reported that HLA-G is expressed in the human thymus raising the possibility that maternal unresponsiveness to HLA-G expressing foetal tissues may be shaped in the thymus by central presentation of this MHC molecule on the medullary epithelium (Crisa *et al.* 1997) HLA-G is known to be capable of stimulating a HLA-G restricted cytotoxic T lymphocyte response and HLA-G molecules can serve as target molecules in lytic reaction with cytotoxic T lymphocytes and HLA-G expressed internally in vivo in transgenic animals is involved in education of the lymphocytic repertoire (Schmidt *et al.*, 1997). The invention shows that the induction of education to foetal antigens occurs during pregnancy and arises from exposure of the mother to foetal antigens during pregnancy. HLA-G allele combinations that were unacceptable in first pregnancy and/or were linked to pre-eclampsia were acceptable in second pregnancy without any associated pregnancy complications. Thus, induction of education to foetal antigens is likely to arise from a process involving HLA-G. Thus the invention offers a means of inducing education including tolerance to HLA-G and/or peptides bound to HLA-G in an individual through mimicking the exposure to foetal antigens that occurs during pregnancy. Thus treatment of an individual with HLA-G and or / peptides known to bind to HLA-G constitutes a means to

induce education in an individual to antigens. In particular, this offers a means to induce tolerance to antigens that cause susceptibility to pre-eclampsia, susceptibility to miscarriage, autoimmune disease and transplant rejection.

In normal pregnancy, direct and indirect alteration of the level and/or activity of molecules arising from the interaction of HLA-G expressing foetal cells with blood mononuclear cells such as lymphocytes and natural killer cells permit pregnancy to progress properly. In pre-eclampsia, miscarriage, miscarriage-related infertility and intrauterine growth retardation and any other HLA-G related disorders, the alteration of the level and/or activity of molecules arising from the interaction of HLA-G and/or HLA-G expressing foetal cells with blood mononuclear cells such as lymphocytes and natural killer cells is likely to be compromised by comparison with that occurring during normal pregnancy. Thus, mimicry of the alteration of the level and/or activity of one or more molecules arising from the interaction of HLA-G and/or HLA-G expressing foetal cells with blood mononuclear cells in an individual constitutes a therapeutic means for prevention and/or treatment of pre-eclampsia, miscarriage, miscarriage-related infertility and intrauterine growth retardation and any other HLA-G related disorders.

The deficit of maternal HLA-G C-93/D-E8 genotypes and the excess of T-93/I-E8 genotypes transmitted to control offspring but not to pre-eclampsia offspring implies selection of fetuses in normal pregnancy dependent on HLA-G genotype. For fertility purposes, and especially in vitro fertilisation and embryo transfer in animals, selection of one or both mating partners, sperm and/or egg donors and/or embryo recipients based on male and/or female HLA-G and/or HLA-G homologue genotypes and/or serotypes and/or activity associated with a successful normal first pregnancy with a specific mating partner offers a means to improve fertility and the success rate of in vitro fertilisation and embryo transfer in animals and improve pregnancy outcome.

Since HLA-G protects trophoblasts from blood mononuclear cell mediated killing, direct and indirect measurement of measurable substances which originate from trophoblast cell killing should allow diagnosis of susceptibility to pre-eclampsia, miscarriage, intra-uterine growth retardation, and monitoring of pregnancy for normal progress, and progress towards pre-eclampsia, miscarriage and intra-uterine growth retardation in humans and animals. More specifically, the interaction between MHC molecules such as HLA-G and blood mononuclear cells is known to directly and indirectly alter the synthesis and levels of several cytokines. Similarly, trophoblasts are known to synthesise and secrete several cytokines. In particular, the altered regulation of some of these cytokines would be expected to compromise the foetal - maternal immune interaction and could be manifest as pre-eclampsia and/or eclampsia and/or intrauterine growth retardation and/or miscarriage and/or miscarriage-related infertility. For example, the interaction of HLA-G expressing cells with blood mononuclear cells increases the amount of interleukin-

3 (IL-3) and interleukin-1 beta (IL-1 beta) and decreases the amount of tumour necrosis factor-alpha (TNF-alpha) release from the blood mononuclear cells. Trophoblasts are known to produce the immunosuppressive cytokine interleukin 10 - a cytokine that potently inhibits alloresponses in mixed lymphocyte reactions. Trophoblasts are also known to produce interleukin 2, a cytokine that both protects the foetus and is involved in activation of maternal killer cells to protect against invading trophoblasts, interleukin 4 and its receptor, which play a role in regulation of umbilical blood flow mediated through the induction of cyclooxygenase-2, indicating a role for interleukin 4 in vascular tone and blood flow modulation during pregnancy, interleukin 6, which is likely to play a role in tissue remodelling associated with placentation.

10 Since the indications are that pre-eclampsia, miscarriage, miscarriage-related infertility and intrauterine growth retardation arise through a HLA-G mediated mechanism, there are several obvious methods for screening for agents which can potentially be used as diagnostic indicators and therapeutic agents. Screening of gene expression profiles using DNA probe arrays allows identification of genes expressed in HLA-G expressing cells and in blood mononuclear cells and genes whose expression changes as a result of HLA-G interaction with blood mononuclear cells. Comparison of the gene expression profile in HLA-G expressing cells and/or blood mononuclear cells and/or HLA-G expressing cells interacting with blood mononuclear cells and/or in blood mononuclear cells interacting with HLA-G allows identification of agents which can potentially be used as diagnostic indicators and therapeutic agents for pre-eclampsia, miscarriage, miscarriage-related infertility and intrauterine growth retardation.

20 HLA-G function and HLA-G expression can be measured. Thus screening for agents which alter the expression and/or function and/or which mimic the function of HLA-G provide a method for screening for potential pre-eclampsia, miscarriage, miscarriage-related infertility and intrauterine growth retardation therapeutic agents.

25 The words "comprises/comprising" and the words "having/including" when used herein with reference to the present invention are used to specify the presence of stated features, integers, steps or components but does not preclude the presence or addition of one or more other features, integers, steps, components or groups thereof.

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Table 1: Genotype and allele distribution of the HLA-G C/T-93 (C1488T) and I/D-E8 (exon deletion) polymorphisms in pre-eclamptic and control offspring.

Polymorphism	genotype	control offspring	preeclamptic offspring	χ^2	p
	<u>Aa</u>	<u>22</u>	<u>44</u>		
<u>HLA-G C/T-93 polymorphism</u>	<u>aa</u>	<u>8</u>	<u>3</u>	<u>6.11</u>	<u><0.02</u>
	<u>AA</u>	<u>18</u>	<u>7</u>	<u>11.01</u>	<u><0.001</u>
	<u>Aa</u>	<u>30</u>	<u>31</u>		
<u>HLA-G C/T-93 polymorphism</u>	<u>aa</u>	<u>12</u>	<u>8</u>	<u>0.69</u>	<u>0.3</u>
	<u>AA</u>	<u>13</u>	<u>12</u>	<u>0.06</u>	<u><0.8</u>

Table 2: Genotype and allele distribution of the HLA-G C/T-93 and I/D-E8 polymorphisms in primigravida trios.

C/T-93					Frequency
	n	C/C (%)	C/T (%)	T/T (%)	C/T
Mothers	90	19(21.1)	50(55.6)	21(23.3)	0.49/0.51
Fathers	90	34(37.8)	39(43.3)	17(18.9)	0.59/0.41
Offspring	90	24(26.7)	41(45.5)	25(27.8)	0.49/0.51
- Male	46	13(28.3)	20(43.4)	13(28.3)	0.5/0.5
- Female	44	11(25.0)	21(47.7)	12(27.3)	0.49/0.51
I/D-Exon 8					Frequency
	n	I/I (%)	I/D(%)	D/D(%)	I/D
Mothers	90	17(18.9)	58(64.4)	15(16.7)	0.51/0.49
Fathers	90	14(15.6)	49(54.4)	27(30.0)	0.43/0.57
Offspring	90	21(23.3)	47(52.2)	22(24.5)	0.49/0.51
- Male	46	10(21.7)	26(56.6)	10(21.7)	0.5/0.5
- Female	44	11(25.0)	21(47.7)	12(27.3)	0.49/0.51

Table 3: Genotype mating outcomes for the HLA-G polymorphisms in control and pre-eclampsia trios.

Mother	Father	Offspring	mt/mnt/pt/pnt	93***	Exon 8****
AA	AA	AA	AAAA	7	4
aa	aa	aa	aaaa	6	3
AA	aa	Aa	AAaa	6	7
aa	AA	Aa	aaAA	8	2
AA	Aa	AA	AAaA	4	1
AA	Aa	Aa	AAaA	2	5
aa	Aa	aa	aaaA	4	8
aa	Aa	Aa	aaAa	3	2
Aa	AA	AA	AaAA	7	7
Aa	AA	Aa	aAAA	12	1
Aa	aa	aa	aAaa	4	3
Aa	aa	Aa	Aaaa	1	14
Aa	Aa	AA	AaAa	6	9
Aa	Aa	Aa	AaaA*	0	10
			aAAa*	3	0
			(Aa)**	6	6
Aa	Aa	aa	aAaA	11	8
total				90	90

5 mt/mnt/pt/pnt = maternally transmitted / maternally non-transmitted / paternally transmitted / paternally non-transmitted. * Allele transmitted assigned from haplotype analysis, ** not possible to determine allele transmitted, ***for C/T-93 matings, A = C-93, a = T-93, ****for I/D-E8 matings, A = I-E8, a = D-E8

Table 4: Relative risk of foetal, maternal and parent of origin effects in a log linear model

	4-factor model		Stepwise reduced model ¹	
<u>Risk Factor</u>	<u>Relative risks</u>			
	C-93	I-E8	C-93	I-E8
1 or 2 alleles in offspring	0.38*	0.43	0.29**	0.39*
1 or 2 alleles in mother	0.85	1.17	----	----
maternal origin	0.70	4.03**	----	4.68***
paternal origin	1.59	0.88	2.12*	----
Chi-square improvement in fit ²	14.6	23.8	12.9	24.5
overall p-value	0.006	0.0001	0.002	0.000005

* significance at the 5% level

** significance at the 1% level

*** significance at the 0.1% level

¹ The model was reduced by stepwise elimination of parameters whose significance was greater than 0.10.

² Compared to a model where none of the four factors shown are fitted i.e. only a term for mating-type stratification is fitted as it is for all models here.

Table 5: Comparisons within primigravida trios.

Maternal transmitted vs. paternal transmitted		
	C/T-93	$p_1=0.009$
	I/D-E8	$p_1=0.000001$
	93-E8 haplotype	$p_3=0.000003$
Maternal non-transmitted vs. paternal non-transmitted alleles		
	C/T-93	$p_1=0.75$
	I/D-E8	$p_1=0.016$
	93-E8 haplotype	$p_3=0.028$
Allele transmitted to offspring		
	C-93 vs. T-93 (transmission disequilibrium test)	$p=0.062$
	I-E8 vs. D-E8 (transmission disequilibrium test)	$p=0.37$
Maternal transmitted vs. non-transmitted alleles		
	C-93 vs. T-93 (transmission disequilibrium test)	$p=0.032$
	I-E8 vs. D-E8 (transmission disequilibrium test)	$p=0.0005$
Paternal transmitted vs. non-transmitted alleles		
	C-93 vs. T-93 (transmission disequilibrium test)	$p=0.87$
	I-E8 vs. D-E8 (transmission disequilibrium test)	$p=0.01$

Probability values (p) are presented with the numbers of degrees of freedom as a subscript.

Table 6: Extended haplotype transmission and frequency.

Haplotype	MT	MNT	PT	PNT	Frequency
polymorphic sites					
31-93-107-110-E8					
a-a-a-a-b	17	30	36	25	0.321
a-b-a-a-a	41	18	18	23	0.298
a-a-a-a-a	6	11	10	19	0.137
a-b-a-a-b	6	16	14	9	0.134
a-a-a-b-b	2	5	2	2	0.032
a-a-b-a-a	4	2	2	2	0.030
a-a-a-b-a	3	0	0	1	0.012
a-a-b-a-b	1	1	0	1	0.009
a-b-b-a-b	0	1	1	1	0.009
a-b-b-a-a	2	0	0	0	0.006
a-b-a-b-b	1	0	1	0	0.006
a-b-a-b-a	1	0	0	0	0.003
a-a-b-b-b	0	0	0	1	0.003

37, 93, 107, 110, and E8 refer to the polymorphic sites in codon 31, 93, 107, 110 and E8. "a" and "b" represent the most common and least common allele respectively of each polymorphic site. MT/MNT/PT/PNT= maternally transmitted / maternally non-transmitted / paternally transmitted / paternally non-transmitted.

Table 7: Transmitted and non-transmitted HLA-G haplotypes to offspring in primigravida trios.

HLA-G Haplotype	Maternal transmitted haplotype	Maternal non-transmitted haplotype	Paternal transmitted haplotype	Paternal non-transmitted haplotype
C-93/I-E8	13 (0.16)	11 (0.13)	11 (0.13)	22 (0.26)
C-93/D-E8	21 (0.25)	38 (0.45)	40 (0.48)	29 (0.34)
T-93/D-E8	6 (0.07)	18 (0.22)	19 (0.22)	9 (0.11)
T-93/I-E8	44 (0.52)	17 (0.20)	14 (0.17)	24 (0.29)
n	84	84	84	84

Table 8: Transmitted and non-transmitted HLA-G haplotypes in trios.

Haplotype MT - PT		Offspring n	Haplotype T - NT		Mothers n	Fathers n
C-I	C-I	3	C-I	C-I	2	3
C-I	C-D	6	C-I	C-D	4	3
C-D	C-I	2	C-D	C-I	2	10
C-I	T-D	2	C-I	T-D	6	1
T-D	C-I	2	T-D	C-I	2	7
C-I	T-I	2	C-I	T-I	1	4
T-I	C-I	4	T-I	C-I	5	2
C-D	C-D	13	C-D	C-D	11	19
C-D	T-D	5	C-D	T-D	3	2
T-D	C-D	0	T-D	C-D	0	3
C-D	T-I	0	C-D	T-I	5	9
T-I	C-D	21	T-I	C-D	23	4
T-D	T-D	4	T-D	T-D	2	3
T-D	T-I	1	T-D	T-I	2	6
T-I	T-D	8	T-I	T-D	7	3
T-I	T-I	11	T-I	T-I	9	5
n =		84			84	84

T: Haplotype transmitted to offspring, NT: Haplotype non-transmitted to offspring.

MT: Haplotype transmitted from mother to offspring, PT: Haplotype transmitted from father to offspring

Table 9: Genotype and allele distribution of the HLA-G C/T-93 and I/D-E8 polymorphisms in pre-eclampsia primigravida trios.

		C/T-93			Frequency	I/D-Exon 8			Frequency
Controls	n	C/C (%)	C/T (%)	T/T (%)	C/T	I/I (%)	I/D(%)	D/D(%)	I/D
Mothers	90	19(21.1)	50(55.6)	21(23.3)	0.49/0.51	17(18.9)	58(64.4)	15(16.7)	0.51/0.49
Fathers	90	34(37.8)	39(43.3)	17(18.9)	0.59/0.41	14(15.6)	49(54.4)	27(30.0)	0.43/0.57
Offspring	90	24(26.7)	41(45.5)	25(27.8)	0.49/0.51	21(23.3)	47(52.2)	22(24.5)	0.49/0.51
PE									
Mothers	79	30(37.9)	36(45.6)	13(16.5)	0.61/0.39	13(16.5)	47(59.5)	19(24.0)	0.46/0.54
Fathers	76	15(19.7)	48(63.2)	13(17.1)	0.51/0.49	19(25.0)	47(61.8)	10(13.2)	0.56/0.44
Offspring	82	18(22.0)	57(69.5)	7(8.5)	0.57/0.43	14(17.1)	55(67.1)	13(15.8)	0.51/0.49

Table 10: Transmitted and non-transmitted HLA-G haplotypes to offspring in control and pre-eclampsia trios.

HLA-G Haplotype	Maternal transmitted haplotype	Maternal non-transmitted haplotype	Paternal transmitted haplotype	Paternal non-transmitted haplotype
Control Trios				
C-93/I-E8	13 (0.16)	11 (0.13)	11 (0.13)	22 (0.26)
C-93/D-E8	21 (0.25)	38 (0.45)	40 (0.48)	29 (0.34)
T-93/D-E8	6 (0.07)	18 (0.22)	19 (0.22)	9 (0.11)
T-93/I-E8	44 (0.52)	17 (0.20)	14 (0.17)	24 (0.29)
n	84	84	84	84
PE Trios				
C-93/I-E8	5 (0.07)	13 (0.18)	12 (0.17)	11 (0.16)
C-93/D-E8	42 (0.60)	27 (0.39)	22 (0.32)	24 (0.35)
T-93/D-E8	4 (0.06)	5 (0.07)	3 (0.04)	11 (0.16)
T-93/I-E8	19 (0.27)	25 (0.36)	33 (0.47)	22 (0.33)
n	70	70	70	68

Table 11: Genotype mating outcomes for the HLA-G polymorphisms in control and pre-eclampsia trios.

Mother	Father	Offspring	mt/mnt/pt/pnt	Control Trios		PE Trios	
				93 [†]	Exon 8 [†]	93 [†]	Exon 8 [†]
AA	AA	AA	AAAA	7	4	4	1
aa	aa	aa	aaaa	6	3	0	0
AA	aa	Aa	AAaa	6	7	8	2
aa	AA	Aa	aaAA	8	2	0	7
AA	Aa	AA	AAAa	4	1	7	5
AA	Aa	Aa	AAaA	2	5	9	4
aa	Aa	aa	aaaA	4	8	4	5
aa	Aa	Aa	aaAa	3	2	9	6
Aa	AA	AA	AaAA	7	7	4	0
Aa	AA	Aa	aAAA	12	1	7	11
Aa	aa	aa	aAaa	4	3	0	4
Aa	aa	Aa	Aaaa	1	14	5	4
Aa	Aa	AA	AaAa	6	9	2	6
Aa	Aa	Aa	AaaA*	0	10	6	3
			aAAa*	3	0	2	5
			(Aa) [†]	6	6	6	7
Aa	Aa	aa	aAaA	11	8	2	3

mt/mnt/pt/pnt = maternally transmitted / maternally non-transmitted / paternally transmitted / paternally non-transmitted. [†]For C/T-93 matings, A = C-93, a = T-93, [†]for I/D-E8 matings, A = I-E8, a = D-E8. * Allele transmitted assigned from haplotype analysis, [†] not possible to determine allele transmitted.

Table 12: Comparisons between Control and pre-eclampsia trios

	Mothers	Fathers	Offspring			
<u>Allele frequency</u>						
C/T-93	$p_1=0.03$	$p_1=0.14$	$p_1=0.18$			
I/D-E8	$p_1=0.37$	$p_1=0.02$	$p_1=0.83$			
93-E8 haplotype frequency	$p_3=0.03$	$p_3=0.008$	$p_3=0.03$			
<u>Genotype distribution</u>						
C/T-93	$p_2=0.05$	$p_2=0.02$	$p_2=0.001$			
I/D-E8	$p_2=0.49$	$p_2=0.02$	$p_2=0.14$			
<u>Deviation from Hardy-Weinberg equilibrium</u>						
	Control Offspring	Control Mothers	Control Fathers	PE Offspring	PE Mother s	PE Fathers
C/T-93	$p_1=0.40$	$p_1=0.29$	$p_1=0.34$	$p_1=0.0002$	$p_1=0.69$	$p_1=0.021$
I/D-E8	$p_1=0.67$	$p_1=0.006$	$p_1=0.29$	$p_1=0.002$	$p_1=0.08$	$p_1=0.027$
<u>Parental transmission to offspring</u>						
	MT	MNT	PT	PNT		
C/T-93	$p_1=0.0007$	$p_1=0.88$	$p_1=0.13$	$p_1=0.25$		
I/D-E8	$p_1=0.00006$	$p_1=0.009$	$p_1=0.00002$	$p_1=0.44$		
93-E8 haplotype	$p_3=0.0002$	$p_3=0.02$	$p_3=0.00003$	$p_3=0.43$		

MT = maternally transmitted, MNT = maternally non-transmitted

PT = paternally transmitted, PNT = paternally non-transmitted

Probability values (p) are presented with the numbers of degrees of freedom as a subscript.

Table 13: Comparisons within control trios and within pre-eclampsia trios.

a) <u>Comparison of transmitted and non-transmitted alleles</u>		Controls	PE
Heterozygote vs. homozygote mating outcome			
	C/T-93	$p_1=0.256$	$p_1=0.002$
	I/D-E8	$p_1=0.317$	$p_1=0.014$
Allele transmitted to offspring			
	C-93 v. T-93 (TDT) table 5		$p=0.49$
	I-E8 v. D-E8 (TDT) table 5		$p=0.77$
Maternal transmitted vs. non-transmitted alleles			
	C-93 v. T-93 (TDT) table 5		$p=0.65$
	I-E8 v. D-E8 table 5		$p=0.09$
	(transmission disequilibrium test)		
Paternal transmitted vs. non-transmitted alleles			
	C-93 v. T-93 table 5		$p=0.60$
	(transmission disequilibrium test)		
	I-E8 v. D-E8 table 5		$p=0.24$
	(transmission disequilibrium test)		
b) <u>Test of difference between parent of origin</u>			
Maternal transmitted vs. paternal transmitted			
	C-93 v. T-93 table 5		$p_1=0.03$
	I-E8 v. D-E8 table 5		$p_1=0.0007$
	93/E8 haplotypes table 5		$p_3=0.005$
Maternal non-transmitted vs. paternal non-transmitted alleles			
	C-93 v. T-93 table 5		$p_1=0.5$
	I-E8 v. D-E8 table 5		$p_1=0.49$
	93/E8 haplotypes table 5		$p_3=0.43$

Probability values (p) are presented with the numbers of degrees of freedom as a subscript.

Table 14: Transmitted and non-transmitted HLA-G haplotypes in control and pre-eclampsia trios.

HLA-G Haplotype	Control Offspring	PE Offspring	HLA-G Haplotype	Control Mothers	Control Fathers	PE Mothers	PE Fathers
MT - PT	n	n	T - NT	n	n	n	n
C-I C-I	3	1	C-I C-I	2	3	0	2
C-I C-D	6	3	C-I C-D	4	3	3	3
C-D C-I	2	2	C-D C-I	2	10	11	1
C-I T-D	2	0	C-I T-D	6	1	0	5
T-D C-I	2	3	T-D C-I	2	7	1	1
C-I T-I	2	1	C-I T-I	1	4	2	2
T-I C-I	4	4	T-I C-I	5	2	1	7
C-D C-D	13	8	C-D C-D	11	19	16	8
C-D T-D	5	3	C-D T-D	3	2	2	1
T-D C-D	0	1	T-D C-D	0	3	0	0
C-D T-I	0	22	C-D T-I	5	9	13	12
T-I C-D	21	9	T-I C-D	23	4	8	13
T-D T-D	4	0	T-D T-D	2	3	1	0
T-D T-I	1	0	T-D T-I	2	6	2	1
T-I T-D	8	0	T-I T-D	7	3	2	5
T-I T-I	11	6	T-I T-I	9	5	8	7
n =	84	63		84	84	70	68

MT: haplotype transmitted from mother to offspring, PT: haplotype transmitted from father to offspring

T: haplotype transmitted to offspring, NT: haplotype non-transmitted to offspring.

Table 15: Relative risk of foetal, maternal and parent of origin effects in a log linear model

Risk Factor	Relative risks	
	PE	
	C-93	I-E8
1 or 2 alleles in offspring	3.51*	1.7
1 or 2 alleles in mother	0.66	0.98
maternal origin	1.12	0.59
paternal origin	0.74	1.11

* significance at the 5% level

Table 16: Genotype and allele distribution of the HLA-G C/T-93 and I/D-E8 polymorphisms in recurrent miscarriage couples.

		C/T-93			Frequency
	n	C/C	C/T	T/T	C/T
Females	22	13	7	2	0.75/0.25
Males	20	6	12	2	0.6/0.4

		I/D-Exon 8			Frequency
	n	I/I (%)	I/D(%)	D/D(%)	I/D
Females	22	3	11	8	0.39/0.61
Males	21	5	12	3	0.55/0.45

	C-93/I-E8	C-93/D-E8	T-93/D-E8	T-93/I-E8
Females	7	25	1	9
Males	7	17	1	15

5 Table 17: HLA-G haplotypes in recurrent miscarriage couples.

Couple no.	Female partner HLA-G haplotype	Male partner HLA-G haplotype
1	t-i / c-d	t-i / c-d
2	c-i / c-d	c-i / c-d
3	c-i / c-d	t-i / c-d
4	c-i / c-d	t-i / c-d
5	c-d / c-d	t-i / c-d
6	c-i / c-d	c-i / c-i
7	c-d / c-d	c-i / t-i
8	c-i / t-i	t-d / c-i
9	t-i / c-d	t-i / t-i
10	t-i / t-i	t-i / c-d
11	c-d / c-d	c-i / c-i
12	c-d / c-d	t-i / c-d
13	t-i / t-i	t-i / c-d
14	t-d / c-i	c-d / c-d
15	c-d / c-d	t-i / t-i
16	c-i / c-d	t-i / c-d
17	c-d / c-d	c-d / c-d
18	c-d / c-d	c-d / c-d
19	t-i / c-d	t-i / c-d
20	t-i / c-d	t-i / c-d

Table 18: Transmitted and non-transmitted HLA-G haplotypes (extended genotypes) in first and second offspring of normal mothers

haplotypes	CMT / MNT	CFT / FNT	first offspring	second offspring
C-I / C-D	2	2	2	0
C-D / C-I	1	2	5	5
C-I / T-D	1	0	0	0
T-D / C-I	0	1	1	1
C-I / T-I	2	5	3	1
T-I / C-I	4	0	0	0
C-D / T-D	3	1	2	0
T-D / C-D	5	3	3	2
C-D / T-I	6	3	0	5
T-I / C-D	2	2	1	5
T-D / T-I	3	3	4	3
T-I / T-D	2	6	6	6
C-I / C-I	0	0	0	0
C-D / C-D	12	14	14	11
T-D / T-D	1	1	1	0
T-I / T-I	4	5	5	8
T-I / T-I	4	5	5	8

MT: haplotype transmitted from mother to offspring, PT: haplotype transmitted from father to offspring

T: haplotype transmitted to offspring, NT: haplotype non-transmitted to offspring.

Table 19: Transmitted and non-transmitted HLA-G extended genotypes in first and second offspring of primigravida pre-eclampsia mothers

	first offspring pre-eclampsia	second offspring normal pregnancy	mother	father
	MT / PT	MT / PT		
1	C-D / C-D	C-D / C-D	C-I / C-D	C-D / C-D
2	T-I / C-D	C-D / C-D	C-D / T-I	C-I / C-D
3	T-I / C-D	T-I / C-D	T-I / T-I	C-D / T-I
4	C-I / C-D	C-D / C-D	C-I / C-D	C-D / T-I
5	C-D / T-I*	C-D / T-I*	C-D / C-D	C-D / T-I
6	C-D / T-I*	C-D / T-I*	C-D / T-D	T-I / T-I
7	C-D / T-I*	C-D / T-I*	C-D / C-D	C-I / T-I
8	T-D / C-I	T-D / C-I	T-D / C-I	C-I / C-I
9	T-I / C-D	T-I / C-D	T-I / T-I	C-D / T-I

MT: haplotype transmitted from mother to offspring, PT: haplotype transmitted from father to offspring

CLAIMS

1. A method for diagnosing susceptibility to normal pregnancy, pre-eclampsia and/or eclampsia and/or intrauterine growth retardation and/or susceptibility to miscarriage and/or miscarriage-related infertility comprising the steps of:

- a) obtaining a fluid and/or tissue sample from a female and/or male and/or foetus; and either
- b) determining the sequence of all or part of the HLA-G nucleic acid, and/or HLA-G linked nucleic acid; or
- c) detecting variant forms of all or part of the HLA-G protein, and/or proteins encoded by HLA-G linked genes or:
- d) measuring the functional activity of all or part of the HLA-G encoding protein and/or proteins encoded by HLA-G linked genes or:
- e) measuring the size and /or level of all or part of HLA-G mRNA or mRNA transcribed from HLA-G linked genes or:
- f) measuring the size and /or level of all or part of HLA-G protein and/or protein encoded by HLA-G linked genes or:
- g) quantifying cells or molecules whose concentration changes as a result of HLA-G action; and
- h) comparing any of the parameters b) to g) with those of a female and/or male and/or foetus of a normal pregnancy and/or a pregnancy with pre-eclampsia and/or eclampsia and/or intrauterine growth retardation and/or susceptibility to miscarriage and/or miscarriage-related fertility outcome.

2. A method as claimed in claim 1 wherein the HLA-G nucleic acid is analysed for the presence of the C and/or T allele of codon 93 in exon 3 and/or the insertion and/or deletion allele of exon 8.

3. A method as claimed in claim 1 wherein the effect of one or more of the HLA-G sequence variants on the functional activity of HLA-G and / or on the size and /or level of all or part of HLA-G mRNA and/or its encoded polypeptide is measured.

4. A method as claimed in claim 1 or 2 wherein all or part of any HLA-G sequence and/or HLA-G linked sequences is amplified, preferably by a method or combination of methods selected from the polymerase chain reaction, nucleic acid sequence based amplification, self sustained sequence replication, transcription-mediated amplification, strand displacement amplification, and the ligase chain reaction.

5. A method as claimed in claim 1-4 wherein comparing of one or more variants identified is performed by association and/or linkage analysis and/or transmission analysis.

6. A method as claimed in any preceding claim wherein all or part of the HLA-G sequence is cloned into a vector.

7. A method as claimed in any preceding claim wherein all or part of the nucleic acid sequence is identified by a method or combination of methods selected from DNA sequencing, glycosylase mediated polymorphism detection, restriction fragment length polymorphism analysis, enzymatic or chemical cleavage analysis, hybridisation to DNA and /or RNA probes and /or DNA probe arrays and/or allele specific DNA and /or RNA probes, allele specific amplification analysis, electrophoretic mobility analysis and 5' nuclease assay analysis.

8. A method as claimed in any preceding claim wherein all or part of HLA-G and /or all or part of one or more variants thereof is expressed as a polypeptide *in vitro* and/or in a prokaryotic and / or eukaryotic cell.

9. A method as claimed in claim 1 wherein the cells of step (g) are blood mononuclear cells and / or T cell and /or natural killer cell subsets thereof and/or HLA-G expressing cells.

10. A method as claimed in any preceding claim wherein the activity of HLA-G and/or any combination of variants thereof and/or blood mononuclear cells and /or a subset of such cells, selected from T cells and/or natural killer cells, is measured by one or more of the following procedures:

- (a) measuring the interaction of HLA-G and /or variants thereof with blood mononuclear cells and/or subsets thereof by assessing one or more of the following with respect to HLA-G expressing cells and /or blood mononuclear cells: cell proliferation, transformation, cytotoxic response, surface marker expression, cytokine production, conjugate formation and target specificity,
- (b) measuring the size and / or level of all or part of HLA-G mRNA and/or its encoded polypeptide,
- (c) measuring the peptide binding capability of all or part of HLA-G and /or variants thereof,
- (d) measuring the binding capability of all or part of the HLA-G and /or variants thereof to a HLA-G receptor,
- (e) measuring one or more molecules whose level is altered as a result of the interaction of the HLA-G and /or variants thereof and /or cells expressing HLA-G with blood mononuclear cells,
- (f) measuring the expression levels of one or more genes and/or proteins in the HLA-G expressing cells.

11. A method as claimed in any preceding claim wherein blood mononuclear cells and/or subsets thereof and/or HLA-G and/or HLA-G linked variants thereof and/or cells expressing all or part of the variants fully and/or partially matching a female and/or male and/or foetus are selected from a test panel.

12. A method as claimed in any preceding claim wherein the HLA-G is partially or fully purified from a cell expressing HLA-G.

13. A method as claimed in any preceding claim wherein the HLA-G is detected by immunoassay using one or more antibodies specific for HLA-G and/or variants thereof.

14. A method as claimed in any preceding claim wherein all or part of the HLA-A, HLA-B, HLA-C, HLA-E, HLA-F and HLA-H genes are analysed in the female and/or male and/or foetus.

15. A method as claimed in claim 1 wherein the molecules of step (g) are selected from IL-1 beta, IL-2, IL-3, IL-4, IL-6, IL-10 and tumour necrosis factor-alpha, or trophoblast specific markers selected from cytokeratins, pregnancy specific glycoprotein 1, human chorionic gonadotrophin and human placental lactogen.

16. A method for screening for agents which can potentially be used as diagnostic indicators and/or drug targets for pre-eclampsia, miscarriage, miscarriage-related infertility and intrauterine growth retardation by:

a) measuring the expression level of one or more genes and/or proteins in HLA-G expressing cells and/or blood mononuclear cells and/or T cell and/or natural killer cells subsets thereof following interaction with HLA-G and/or HLA-G expressing cells;

b) comparing the expression level identified in step a) with the expression level in HLA-G expressing cells and/or the blood mononuclear cells and/or T cell and/or natural killer cell subsets thereof following interaction with HLA-G and/or HLA-G expressing cells in normal pregnancy and/or pre-eclampsia pregnancy and/or intrauterine growth retardation pregnancy and/or miscarriage pregnancy and/or miscarriage-related infertility.

17. A method as claimed in claim 10 or 16 wherein gene expression and/or protein expression is measured by any one or combination of methods selected from hybridisation between cDNA and/or RNA from the cells and DNA probes and/or RNA probes and/or nucleic acid probe arrays, quantitative amplification methods, reverse transcriptase - polymerase chain reaction (RT-PCR), 5' nuclease assay, ribonuclease protection assay and S1 nuclease assay, one dimensional and/or two dimensional gel electrophoresis and staining of proteins, detection of one or more proteins using, enzyme linked immunosorbent assays (ELISA), radioimmunoassays (RIA), protein truncation test (PTT), immunoradiometric assays (IRMA) and immunoenzymatic assays (IEMA), sandwich assays and Western blotting using monoclonal and/or polyclonal antibodies.

18. A pharmaceutical composition comprising a pharmaceutically effective amount of HLA-G and/or cells expressing HLA-G and/or one or more peptides which binds to HLA-G, blood mononuclear cells from a donor and/or test panel known to interact with HLA-G variants, cytokines and any combination thereof including IL-1 beta, IL-2, IL-3, IL-4, IL-6, IL-10 and tumour necrosis factor-alpha and/or

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inhibitors of cytokines and/or tumour necrosis factor alpha and/or derivatives of cytokines and/or tumour necrosis factor-alpha, optionally with pharmaceutically-acceptable carriers or excipients.

19. Use of HLA-G or HLA-G expressing genes in a method of screening for potential therapeutic agents for the treatment of a condition selected from:- pre-eclampsia, eclampsia, intrauterine growth retardation, susceptibility to miscarriage and miscarriage-related infertility.

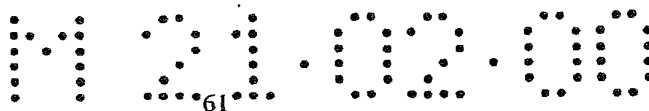
20. A method for the prevention of pre-eclampsia and/or eclampsia and/or intrauterine growth retardation and/or susceptibility to miscarriage and/or miscarriage-related infertility selected from:

- a) treatment of a female with all or part of a pharmaceutically effective amount of a HLA-G and /or peptides which bind to HLA-G and / or cells expressing HLA-G;
- b) treatment of a female with all or part of a pharmaceutically effective amount of molecules or inhibitors of molecules whose level or activity is directly or indirectly altered by HLA-G action;
- c) treatment of a female with all or part of a pharmaceutically effective amount of molecules which inhibit the interaction between HLA-G and one or more of its receptors;
- d) treatment of a female with all or part of a pharmaceutically effective amount of an agent which alters HLA-G expression;
- e) treatment of a female with all or part of a pharmaceutically effective amount of an agent which alters HLA-G related blood mononuclear cell activity;
- f) treatment of a female with all or part of a pharmaceutically effective amount of an agent which mimics all or part of HLA-G action;
- g) treatment of a female with blood mononuclear cells that recognise foetal and / or self HLA-G;
- h) treatment of a female with HLA-G and / or cells expressing HLA-G or variants thereof;
- i) treatment of a female with one or more antibodies which bind to HLA-G and / or cells expressing HLA-G and / or any receptor for HLA-G;
- j) introduction of one or more variants of the HLA-G gene and /or its receptor into a female and / or male;
- k) introduction of an inhibitor of expression of the HLA-G gene and/or its receptor into a female and/or male;
- l) inactivation of one or more variants of the HLA-G gene and/or its receptor in a female and/or male.

21. A method for improving pregnancy success selected from:

- a) pre-treating the female with sperm and/or attenuated forms thereof, and/or semen and/or fractions thereof from a male with a known HLA-G genotype, prior to mating with a male of a different HLA-G

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genotype, and/or in vitro fertilisation using sperm from a male of a different HLA-G genotype and/or embryo transfer where the male HLA-G is of a different HLA-G genotype;

b) mixing sperm of a known HLA-G genotype with sperm and/or attenuated forms thereof, and/or semen and/or fractions thereof from a male with a different HLA-G genotype prior to in vitro fertilisation.

- 5 22. A method as claimed in claim 21 wherein fertility and / or pregnancy outcome are improved by selection of male and / or female partners and / or sperm and / or ova and / or recipients of fertilised eggs and / or zygotes / and / or embryos so that (a) their HLA-G and /or HLA genotypes and /or serotypes or (b) the activity of their HLA-G and / or blood mononuclear cells interacting with HLA-G are indicative of normal pregnancy outcomes and / or not associated with pre-eclampsia and/or eclampsia and/or
- 10 intrauterine growth retardation and/or susceptibility to miscarriage and/or miscarriage-related infertility.

23. A test kit for the diagnosis of susceptibility to normal pregnancy, pre-eclampsia and/or eclampsia and/or intrauterine growth retardation and/or susceptibility to miscarriage and/or miscarriage-related infertility or for monitoring progress of pregnancy comprising:

- 15 a) oligonucleotide primers for amplification of all or part of the HLA-G gene and /or HLA-G linked DNA;
- b) amplification reagents for amplification of genomic DNA and / or RNA segments, selected from a DNA / RNA polymerase, a reverse transcriptase, the deoxyribonucleotides dATP, dCTP, dGTP, dTTP and dUTP, and /or ribonucleotides ATP, CTP, GTP, TTP and UTP, and reaction buffer;
- 20 c) reagents for identifying sequence variants in DNA and / or RNA;
- d) control DNA and /or RNA.

24. Use of a DNA sequence selected from any one of Sequence I.D.s 1 to 21 for diagnosis of susceptibility to or in a test kit for the diagnosis of susceptibility to normal pregnancy, pre-eclampsia and/or eclampsia and/or intrauterine growth retardation and/or susceptibility to miscarriage and/or miscarriage-related infertility, for monitoring progress of pregnancy, for use in the manufacture of a
- 25 medicament, in a method for screening potential therapeutic agents, in a method for screening for potential diagnostic indicators and/or drug targets, in a method for improving pregnancy success or in a method for the prevention of pre-eclampsia and/or eclampsia and/or intrauterine growth retardation and/or susceptibility to miscarriage and/or miscarriage-related infertility, for monitoring progress of pregnancy.

- 25 25. A method for induction of tolerance in a host to a non-self tissue which comprises administering
- 30 HLA-G and /or HLA-G loaded with peptides from the non-self tissue and /or HLA-G expressing cells derived from or related to the non-self tissue, and/or a non-self tissue bearing an introduced HLA-G so that HLA-G is expressed in all or part of the tissue.

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26. A method for the treatment of autoimmune disease which comprises administering HLA-G and /or HLA-G loaded with peptides from a self and/or non-self tissue and / or with specific autoimmune antigen and /or HLA-G expressing cells from a self and/or non-self tissue and/or a self and/or self tissue bearing an introduced HLA-G gene so that HLA-G is expressed in all or part of the tissue.

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SECRET

I hereby claim the benefit under 35 U.S.C. Section 119(e) of any United States provisional application(s) listed below:

(Application Serial No.)

(Filing Date)

(Application Serial No.)

(Filing Date)

(Application Serial No.)

(Filing Date)

I hereby claim the benefit under 35 U.S.C. Section 120 of the United States application(s), or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark office all information known to me to be material to patentability as defined in Title 37, C.F.C., Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

PCT/E99/00012
(Application Serial No.)

25 February 1999
(Filing Date)

Pending
(Status)
(patented, pending, abandoned)

(Application Serial No.)

(Filing Date)

(Status)
(patented, pending, abandoned)

(Application Serial No.)

(Filing Date)

(Status)
(patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Declaration and Power of Attorney for Patent Application English Language Declaration

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

HLA LINKED PRE-ECLAMPSIA AND MISCARRIAGE SUSCEPTIBILITY GENE

the specification of which

(check one)

- ☒ corresponds to PCT/IE99/00012, filed February 25, 1999.
☐ was filed on _____ as United States Application No. or PCT
 Application No. _____
 and was amended on _____
 (if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed

Prior Foreign Application(s)	<u>Priority Not Claimed</u>
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I hereby claim the benefit under 35 U.S.C. Section 119(e) of any United States provisional application(s) listed below:

(Application Serial No.)

(Filing Date)

(Application Serial No.)

(Filing Date)

(Application Serial No.)

(Filing Date)

I hereby claim the benefit under 35 U.S.C. Section 120 of the United States application(s), or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark office all information known to me to be material to patentability as defined in Title 37, C.F.C., Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

PCT/IE99/00012

(Application Serial No.)

25 February 1999

(Filing Date)

Pending

(Status)
(patented, pending, abandoned)

(Application Serial No.)

(Filing Date)

(Status)
(patented, pending, abandoned)

(Application Serial No.)

(Filing Date)

(Status)
(patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

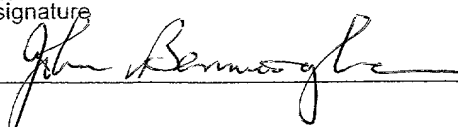
POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. *(list name and registration number)*

David G. Conlin	Reg. No. 27,026	Christine C. O'Day	Reg. No. 38,256
George W. Neuner	Reg. No. 26,964	Robert L. Buchanan	Reg. No. 40,927
Linda M. Buckley	Reg. No. 31,003	David E. Tucker	Reg. No. 27,840
Peter J. Manus	Reg. No. 26,766	Lisa Swiszc Hazzard	Reg. No. 44,368
Peter F. Corless	Reg. No. 33,860	George W. Hartnell	Reg. No. 42,639
Cara Z. Lowen	Reg. No. 38,227	Jennifer K. Holmes	Reg. No. 46,778
William J. Daley, Jr.	Reg. No. 35,487	Kerri Pollard Schray	Reg. No. 47,066

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Fifth inventor's signature <i>Tommie Mc Carthy</i>	Date:
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Ireland	
Post Office Address	
Same As Above	

POWER OF ATTORNEY. As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. (list name and registration number)

David G Conlin	Reg. No. <u>27,026</u>	Christine C. O'Day	Reg. No. <u>38,256</u>
George W Neuner	Reg. No. <u>26,964</u>	Robert L. Buchanan	Reg. No. <u>40,927</u>
Linda M Buckley	Reg. No. <u>31,003</u>	David E. Tucker	Reg. No. <u>27,840</u>
Peter J Manus	Reg. No. <u>26,766</u>	Lisa Swisocz Hazzard	Reg. No. <u>44,368</u>
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William J Daley, Jr	Reg. No. <u>35,487</u>	Kerri Pollard Schray	Reg. No. <u>47,066</u>

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 (name and telephone number)

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 Telephone: (617) 523-3400
 Facsimile (617) 523-6440

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Sole or first inventor's signature <u>Margaret O'Brien</u>	Date: <u>2/11/2000</u>
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Citizenship <u>Ireland</u>	
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Citizenship <u>Ireland</u>	
Post Office Address <u>Same As Above</u>	

3-00

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Third inventor's signature	Date:
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Ireland	
Post Office Address	
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4-00

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Fourth inventor's signature	Date:
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Citizenship	
Great Britain	
Post Office Address	
Same As Above	

5-00

Full name of fifth inventor	
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Fifth inventor's signature	Date:
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Citizenship	
Ireland	
Post Office Address	
Same As Above	

Declaration and Power of Attorney for Patent Application

English Language Declaration

As a below named inventor, I hereby declare that.

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

HLA LINKED PRE-ECLAMPSIA AND MISCARRIAGE SUSCEPTIBILITY GENE

the specification of which

(check one)

- ☒ corresponds to PCT/IE99/00012, filed February 25, 1999.
☐ was filed on _____ as United States Application No. or PCT
 Application No. _____
 and was amended on _____
 (if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed

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